



## **The dark side of innate immune memory: the development of atherosclerosis**

Siroon Bekkering

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## **Colofon**

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# The dark side of innate immune memory: the development of atherosclerosis

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Like love, there can never be too much good music

Haruki Murakami

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# INTRODUCTION

General introduction, aim and outline of the thesis

## GENERAL INTRODUCTION

### A historical perspective

Already in 200 AD, the famous doctor Claudius Galenus from Pergamon (131-201 AD) was the first to describe the heart and arteries<sup>1</sup>. It was only in 1628, almost 1500 years later, that the circulation of blood was described by William Harvey<sup>2</sup>. It was Leonardo da Vinci however with his comprehensive approach who first described arteriosclerosis (or atherosclerosis, which was the same at that time). He observed that 'vessels in the elderly restrict the transit of blood through thickening of the tunics'<sup>3</sup>. His study consisted of three separate fields of research. First of all, he conducted a study of hydrodynamics, studying the flow of fluids in an experimental setup (shear stress and laminar flow). Secondly, he observed the effects of age on anatomical structures, in particular on blood vessels. Thirdly, he described the concept of exercise and nutrition influencing

the process of thickening of the arterial wall. Figure 1 shows the results of his anatomical study, comparing the straightness of the vessels in the young and the tortuosity and thicker coats of the vessels in the elderly. He comments that 'Vessels which by the thickening of their coats in the old restrict the transit of the blood, and from this lack of nourishment the old, little by little with a slow death destroy their life without any fever; and this happens through the lack of exercise since the blood is not warmed' and 'The walls of these tortuous blood vessels are nourished by the vital humour, the blood which they contain; forming as it were the sheath of that moving warm blood they obtain an undue proportion of the nutriment contained in the blood. It is this disproportionate nutrition, prolonged over many years, that eventually leads to the undue growth in thickness of the walls'<sup>4</sup>. Interestingly, several hundred years later, we are still studying the same topics in the field of cardiovascular disease.



*Figure 1: Leonardo da Vinci depicts the contrast between straightness of vessels in the young and their tortuosity in the old*

## Cardiovascular disease

Cardiovascular diseases (CVD) are the leading cause of mortality worldwide, causing 17 million deaths per year<sup>5</sup>. The main underlying pathological process of CVD is atherosclerosis, a chronic inflammatory disorder of the arterial vessel wall, which can ultimately result in vascular occlusion and subsequent myocardial infarction or stroke. Although previously considered a passive cholesterol storage disease of the arterial wall, vascular inflammation is now recognized as the major driver of initiation, progression and rupture of the atherosclerotic plaque<sup>6</sup>. Studies in animal models of atherosclerosis, and novel imaging techniques that can be used in humans in vivo, such as <sup>18</sup>FluoroDeoxyGlucose-Positron Emission Tomography (<sup>18</sup>FDG-PET) scanning have revealed that chronic low-grade inflammation of the arterial wall is an important mechanism contributing to the development of atherosclerosis<sup>7</sup>. Currently, large clinical trials are being performed in patients at risk for CVD with anti-inflammatory agents, including the interleukin-1-receptor antagonist anakinra, the antibody against IL-1b canakinumab, and methotrexate<sup>6</sup>. However, these drugs are inhibiting immune pathways rather broadly and can have serious side effects. Therefore, a better understanding of the inflammatory mechanisms in atherosclerosis is needed to identify novel drug targets that can be used in the prevention and treatment of atherosclerosis.

## The role of monocytes and macrophages in atherosclerosis

Atherosclerotic plaque formation is initiated by the accumulation of apoB-100 containing lipoproteins within the arterial wall. This occurs mainly at those points where the laminar flow is disturbed (da Vinci's first study), which impairs endothelial function and increases endothelial permeability for lipoproteins<sup>8</sup>. These lipoproteins are susceptible to oxidation or other modifications in the intimal layer, which also contributes to further activation of the overlying endothelium, resulting in an upregulation of adhesion molecules and the secretion of chemokines. Subsequently, the intimal layer is infiltrated with various immune cells, including monocytes. A pivotal role for monocytes in the initiation of atherosclerosis was demonstrated by the observation that atherosclerosis development is reduced in animal models by prevention of monocyte entry into the vascular wall<sup>9</sup>. Once in the intimal layer, monocytes mature into macrophages, which promote the development of atherosclerosis by several mechanisms (Figure 2). Firstly, macrophages become cholesterol-laden foam cells by taking up modified lipoproteins via scavenger receptors. This mechanism has been described as a double-edged sword, since initially, clearance of cholesterol by macrophages can be viewed as beneficial, cleaning the vessel wall from the overload of lipids. However, cholesterol-laden macrophages lose their ability to emigrate out of the plaque, and accumulate. Eventually, foam cells die and contribute to the formation of a necrotic core, which is a key component of advanced plaques<sup>6,10</sup>. Secondly, macrophages produce various

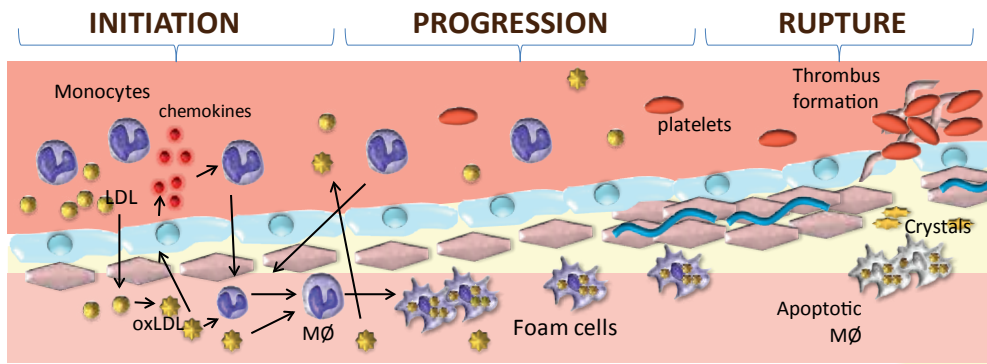


Figure 2: the role of monocytes and macrophages in the different stages of atherosclerosis (Adapted from Clinical Therapeutics Vol 37(4), Bekkering et al. 'The epigenetic memory of monocytes and macrophages as a novel drug target in atherosclerosis', 2014, 914-923, with permission from Elsevier)

pro-inflammatory cytokines and chemokines in response to pro-inflammatory stimuli in the plaque. Modified LDL particles (such as oxidized LDL) can act as Damage Associated Molecular Patterns (DAMPs), recognized by membrane-bound and intracellular Pattern Recognition Receptors (PRRs) on monocytes and macrophages<sup>11</sup>. For example, oxLDL stimulates secretion of interleukin-6 (IL-6), IL-8, monocyte chemo-attractant protein-1 (MCP-1) and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) by signaling through CD36, Toll-like Receptor (TLR) 2, -TLR4 and/or TLR6 pathways<sup>12–14</sup>. Moreover, intracellular and extracellular cholesterol crystals can activate the NLRP3 inflammasome, thereby inducing processing and release of interleukin-1 $\beta$ <sup>14</sup>. Thirdly, macrophages can contribute to destabilization of plaques by the production of many proteases, including various matrix metalloproteinases (MMP). By all these mechanisms, macrophages contribute to the initiation, progression and destabilization of atherosclerotic plaques<sup>15</sup>.

Although the role of monocytes and macrophages in atherosclerosis is well established, it is still unknown why the strong inflammatory response in the arterial wall persists and fails to resolve. In this thesis I propose that 'training' of monocytes can result in a long-term pro-inflammatory phenotype, which contributes to the persistent pro-inflammatory state.

## Trained immunity

Recently, the traditional view on the division between the innate and adaptive immune system has changed, as cells of the innate immune system also appeared to possess adaptive characteristics<sup>16</sup>. Plants and invertebrate species, which lack adaptive immunity, are able to build up an immunological memory and protection against reinfection, suggesting a memory function for innate immunity<sup>17,18</sup>. Recent studies have shown that also in vertebrates, innate immune cells display persistent adaptive characteristics leading to a non-specific

immunological memory, which has been termed ‘trained innate immunity’<sup>16</sup>. A recent series of experiments demonstrated the existence of trained innate immunity in rodents and humans and provided insight into the underlying molecular and cellular mechanism. In isolated human monocytes, brief exposure to various microbial products, including Bacille-Calmette Guérin (BCG) vaccine, *Candida albicans*, or its cell-wall component  $\beta$ -glucan induces a long-lasting pro-inflammatory macrophage phenotype that is characterized by increased production of cytokines and chemokines upon TLR stimulation six days after the initial exposure (Figure 3)<sup>19,20</sup>. Also in healthy humans, BCG-vaccination induces an augmented production of pro-inflammatory cytokines (e.g.  $\text{TNF}\alpha$  and IL-6) when monocytes were subsequently exposed to *M. tuberculosis* ex vivo, but also to various unrelated microbial products<sup>20</sup>. This enhanced pro-inflammatory phenotype of the monocyte can be detected even three months after BCG vaccination. The powerful immunological effects of this phenomenon are illustrated by the findings that in mice that lack T and B lymphocytes, BCG vaccination provides robust protection against a subsequent lethal dose of *Candida albicans*<sup>20</sup>.

It is likely that trained immunity evolved as a primitive form of immune memory, aimed to provide additional protection of the host against reinfection, with increased survival, especially in the newborn, who lack adaptive immunity. However, there may also be a

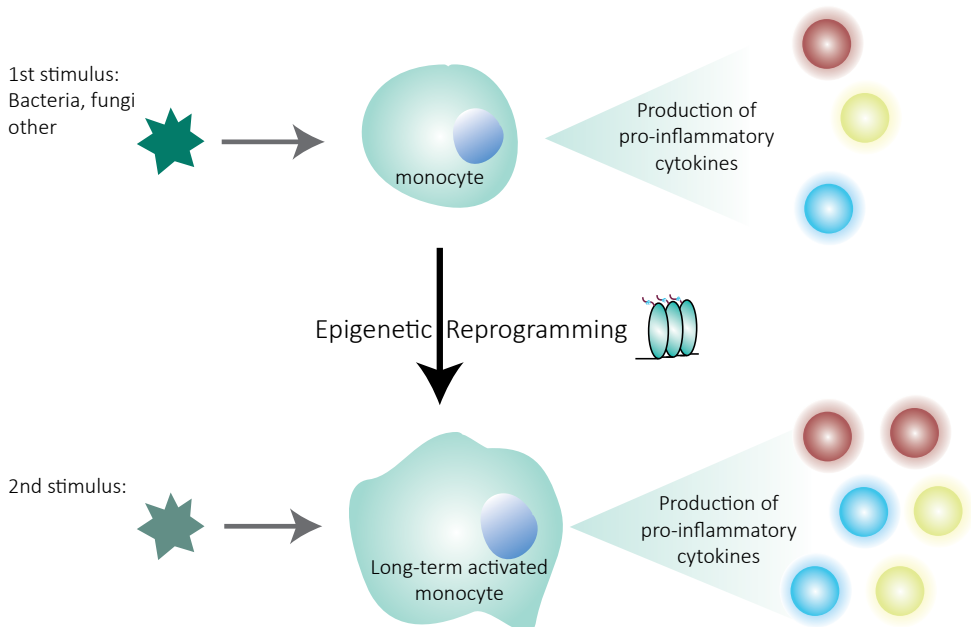


Figure 3: Trained immunity, a schematic overview ©Siroon Bekkering

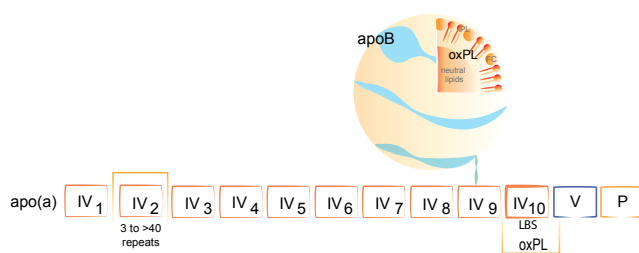
dark side to the reprogramming of innate immunity, in which increased inflammatory responses towards exogenous or endogenous stimuli could have deleterious effects, such as atherosclerosis. Long-term activation of monocytes by exogenous or endogenous triggers could lead to an enhanced immune activation, which can accelerate the process of atherosclerosis. In patients with chronic inflammatory diseases, such as rheumatoid arthritis, there is an increased risk for the development of atherosclerosis, indicating that chronic systemic inflammation may accelerate the process of atherosclerosis. The present thesis is focused on the potential pro-atherosclerotic effects of trained innate immunity.

Since several microorganisms and microbial products have been shown to induce trained immunity, this phenomenon might explain the potential association between infections and atherosclerosis that has been reported in the literature. A variety of microorganisms have been linked to an increased risk of cardiovascular disease, carotid plaque thickness and stroke, including *C. pneumoniae*, *H. pylori*, cytomegalovirus and herpes simplex virus<sup>21–23</sup>. However, the strength of these data associating pathogens with atherosclerosis varies considerably and up till now, the mechanism underlying this association is unclear<sup>24</sup>. In theory, training of monocytes by these microorganisms could be an underlying mechanism linking infection to atherogenesis.

However, it is important to realize that induction of trained immunity does not have to be restricted to microbial products. As described earlier, oxLDL and cholesterol crystals are able to induce an inflammatory response in monocytes by stimulation of various PRRs. Another endogenous compound that is associated with atherosclerosis and with systemic inflammation, is lipoprotein (a). We propose that these endogenous pro-atherogenic compounds can also induce trained innate immunity.

## Lipoprotein(a)

In the general population, approximately 20% of the people have elevated circulating levels of lipoprotein(a) [Lp(a)]. Lp(a) is a plasma lipoprotein composed of an LDL particle with an apolipoprotein(a) [apo(a)] moiety covalently bound to



*Figure 4: Lipoprotein(a) consists of an apoB particle covalently bound to an apo(a) particle*

apolipoprotein B-100 (apoB) (Figure 4). Epidemiological studies, including genome-wide association and Mendelian randomization studies show a strong, independent and likely causal relationship between levels of Lp(a) and the occurrence of myocardial infarction, stroke, peripheral artery disease and calcific aortic valve stenosis<sup>25,26</sup>. Over the last decade, a large body of in vitro and in vivo evidence has shown that Lp(a) is the main carrier of phosphocholine (PC)-containing oxidized phospholipids (OxPL) in plasma. This has led to the hypothesis that a major component of the risk mediated by Lp(a) is through its content of OxPL. OxPL is recognized as a DAMP by PRRs on innate immune cells, similar to oxLDL, leading to a wide range of pro-inflammatory and plaque destabilizing processes. Here we further studied the link between Lp(a), its oxPLs and inflammation by studying the induction of trained innate immunity by Lp(a) in vivo and in vitro.

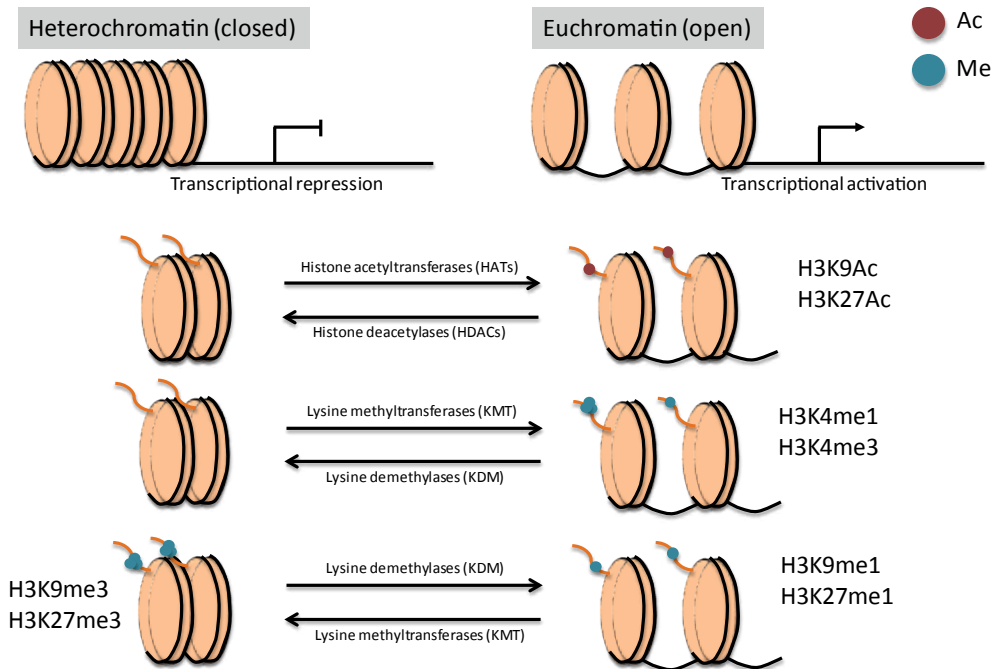
## Mechanisms of trained immunity

The key mechanisms underlying the phenotype of trained innate immunity are a shift in the intracellular metabolism from oxidative phosphorylation towards an increased aerobic glycolysis (ie. the Warburg effect) and epigenetic reprogramming at the level of histone methylation<sup>27</sup>.

Epigenetic regulation comprises the control of gene expression without altering the DNA sequence itself, by posttranslational modifications of the DNA (DNA methylation), of histones, or by non-coding RNA. In general, DNA hypermethylation is associated with transcriptional silencing of genes, whereas DNA hypomethylation is associated with transcriptional activation<sup>28</sup>. Modifications of histones can either activate or repress gene transcription. Histone modifications can directly alter the chromatin structure, thereby facilitating or inhibiting the binding of transcription factors. In general, acetylation of histones neutralizes the positive charge of the involved lysine-residues, which weakens the association between the DNA and the histones, facilitating binding of transcriptional factors and stimulating transcriptional activation<sup>29</sup>. Histone methylation can either lead to transcriptional activation or repression, depending on the specific lysine residue that is modified and the amount of methyl-groups that is added. For example, monomethylation or trimethylation of lysine 4 at histone 3 (H3K4Me1/3) leads to transcriptional activation, whereas H3K9me3 and H3K27Me3 are associated with gene silencing<sup>30</sup> (Figure 5). The different histone modifications can influence each other and can also modulate DNA methylation, in part through the activities of protein complexes that bind modified histones or methylated cytosine residues<sup>31</sup>.

Chromatin immunoprecipitation studies revealed that the trained monocyte is characterized by a global increase in H3K27Ac and H3K4me3, mainly on the promoter region of genes involved in immune defense, signaling, and metabolism, and by an increase of H3K4me1





*Figure 5: Chromatin modifications: Several histone marks can lead to either opening of the chromatin (euchromatin) or closing of the chromatin (heterochromatin). Histone acetylation by histone acetyltransferases always leads to opening of the chromatin, whereas for histone methylation, the location and amount of methylgroups direct opening or closing of the chromatin. Several enzymes are known for adding or removing acetyl and methylgroups to the histone tails. © Siroon Bekkering*

on enhancer regions<sup>32</sup>. The pro-inflammatory phenotype of b-glucan-trained cells was prevented in vitro by co-administration of the nonspecific histone methyltransferase inhibitor methylthioadenosine, suggesting that the increase in histone methylation is responsible for the observed change in phenotype<sup>19</sup>. The kinetics of the epigenetic process leading to this increased functional state of the monocyte and macrophages were recently described by another group<sup>33</sup>. In terminally differentiated cells, Ostuni et al. demonstrated the presence of several regions of the genome that are unbound by transcription factors and lack the histone marks characteristic of enhancers but acquire these features in response to stimulation, which they named 'latent enhancers'<sup>33</sup>.

Another key mechanism involved in the development of trained immunity is cellular metabolism of innate immune cells. The metabolic state of immune cells is a topic of high interest in the past few years<sup>34,35</sup>. It has now been established for various cells of the immune system, such as T-cells or macrophages, that the immunological phenotype is determined by the cellular metabolism. Resting immune cells are mainly relying on the TCA cycle and OXPHOS for energy generation<sup>35</sup>. Upon activation, both T-cells and M1 macrophages immediately

switch to aerobic glycolysis, a phenomenon called the 'Warburg effect', which was first observed in cancer cells. M2 macrophages however, still rely on the OXPHOS upon activation<sup>36</sup>.

Recent studies from our laboratory have reported that a similar metabolic shift occurs in the context of trained immunity. In trained immunity, (microbial) triggers induce a metabolic shift from oxidative phosphorylation to aerobic glycolysis via activation of the mammalian target of rapamycin (mTOR) and its effector hypoxia-inducible factor HIF1 $\alpha$ <sup>27</sup>. Pharmacological blockade of glycolysis completely prevents trained immunity, suggesting that the shift to glycolysis plays a crucial role in the induction of epigenetic reprogramming of the monocyte. The link between this metabolic shift and epigenetic reprogramming is most likely explained by the fact that many intermediate metabolites function as essential cofactors for epigenetic enzymes<sup>27</sup>.

## AIM AND OUTLINE OF THIS THESIS

The aim of this thesis was to investigate whether trained innate immunity can also be induced by endogenous pro-atherogenic substances, such as oxLDL and lp(a), and whether this mechanism plays a role in the process of atherosclerosis (Figure 6). In **Chapter 2**, the hypothesis that trained immunity contributes to the development of atherosclerosis and the potential underlying mechanisms are discussed in depth.

### Trained immunity in atherosclerosis in vitro

In **Chapter 3**, an in vitro model of trained immunity is explored, studying the temporal characteristics and kinetics of trained immunity in the laboratory. We exposed isolated monocytes from healthy subjects to different stimuli, including  $\beta$ -glucan, BCG and oxLDL and characterized the trained immunity phenotype by measuring ex vivo cytokine production, ROS production and lactate production as a measurement of glycolysis. We investigated the effect of different training duration and resting times on the trained immunity phenotype. In **Chapter 4**, the induction of trained immunity by oxidized LDL is explored, studying the effects of brief exposure to a low dose of oxLDL on cytokine production, RNA expression, epigenetic remodeling and foam cell formation. Also, the intracellular pathways of oxLDL induced trained immunity were explored.

### Trained immunity in atherosclerosis in vivo

In **Chapter 5**, we studied whether lipoprotein(a) [lp(a)] also induces trained immunity, both in vitro as well as in patients with elevated levels of lp(a). In these patients, we characterized the phenotype of circulating monocytes, and this was correlated with vascular wall inflammation, that was quantified by use of sophisticated imaging modalities. In **Chapter 6**, we performed a first proof-of-concept study on trained immunity in patients with established atherosclerosis. We isolated monocytes to investigate the trained immune phenotype in patients with severe symptomatic coronary atherosclerosis and matched controls. Furthermore, we explored RNA expression levels of inflammatory mediators and intracellular signaling pathways involved in metabolism, and we explored the epigenetic landscape of the cytokine TNF $\alpha$  in detail for activating and repressing histone marks.

### Pathways of trained innate immunity

In **Chapter 7**, we explored the role of the cholesterol synthesis pathway in trained immunity. By using statins and other specific pharmacological inhibitors, we unraveled a role for mevalonate in the induction of trained immunity, for both  $\beta$ -glucan training as well as oxLDL training. Furthermore, we studied monocytes from patients with a defective

cholesterol synthesis pathway, which accumulate mevalonate. Monocytes from these patients showed a trained immunity phenotype by increased cytokine response upon stimulation and an increased expression of genes in the glycolysis and mTOR pathway.

In **Chapter 8**, we integrate the insights we have gained into the role of trained immunity in the context of atherosclerosis, and speculate on the potential clinical relevance of this mechanism for the diagnosis and treatment of patients with (risk factors for) atherosclerosis. Moreover, we propose a hypothesis of diet-induced trained immunity as one of the mechanisms of chronic inflammation in atherosclerosis.

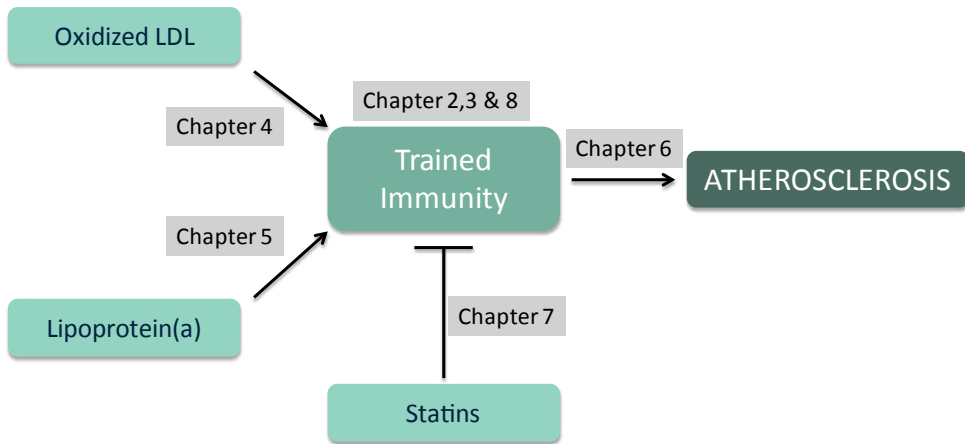


Figure 6: Schematic overview of the thesis

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# CHAPTER 2

## TRAINED INNATE IMMUNITY AND ATHEROSCLEROSIS

**Bekkering S**, Joosten LAB, van der Meer JWM, Netea MG, Riksen NP

Curr Opin Lipid (2013) 24(6):487-492



## **ABSTRACT**

### **Purpose of the review**

Monocytes/macrophages play a decisive role in the development and progression of atherosclerosis. It is currently unknown what stimuli initiate and orchestrate the activation of these cells in atherogenesis. In this review, we postulate that the novel concept of ‘trained immunity’ modulates the development and progression of atherosclerosis.

### **Recent findings**

Recently, results from our laboratory challenged the current paradigm that innate immunity is static and does not have an immunological memory. Stimulation by various microbial products, including *Candida albicans* and bacille Calmette-Guérin, appeared to bring monocytes into a long-term enhanced functional state, showing a stronger pro-inflammatory response to a second stimulus. This ‘trained immunity’ was mediated by increased and stable histone methylation.

### **Summary**

We describe the hypothesis that this functional reprogramming of monocytes, either by microbial products or by metabolic products, contributes to atherogenesis and propose epigenetic reprogramming of monocytes as a novel pharmacological target for preventing or treating atherosclerosis in the future.

## INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of mortality worldwide, causing 17 million deaths per year [1]. The main underlying pathological process of CVD is atherosclerosis, a chronic disorder of the arterial wall, which can ultimately result in vascular occlusion and subsequent myocardial infarction or stroke. Over the past decades, the view on the pathogenesis of atherosclerosis has changed from predominantly being a lipid storage disease to a chronic inflammatory disease of the vascular wall [2]. Atherosclerotic plaques are characterized by an accumulation of lipids and an infiltration with various immune cells, macrophages being the most numerous [2]. Recently, it has been reported by our laboratory that monocytes/macrophages can build up a long-term memory after microbial stimulation via epigenetic reprogramming; this mechanism was termed ‘trained immunity’ [3-5]. In the current review, we postulate that trained immunity contributes to atherogenesis and describe potential novel therapeutic targets to prevent or treat atherosclerosis based on this concept.

### The role of monocytes in the pathogenesis of atherosclerosis

Atherosclerotic inflammation is initiated by accumulation of apolipoprotein B (ApoB) containing lipoproteins, including LDL, in the intimal layer at sites of reduced shear stress. Upon enzymatic oxidative modification, LDL particles activate the endothelium to express more adhesion molecules and enhance the production of chemo-attractants, thereby recruiting monocytes [6]. Prevention of monocyte entry by blocking either chemokines or their receptors prevents or retards atherogenesis in mouse models [7-9], indicating a pivotal role for monocytes in the early development of atherosclerosis. In the intimal layer, the monocytes differentiate into macrophages, a process driven by endothelium-derived macrophage colony-stimulating factor (M-CSF) [10]. Macrophages in the intimal layer subsequently engulf oxidized LDL particles (oxLDL) through scavenger receptors, notably the CD36 and Scavenger Receptor-A (SR-A) and accumulate lipid droplets in the cytoplasm to become “foam cells”; the latter cells play a central role in the formation of the atherosclerotic plaque [11]. In addition, inflammatory activation of lesional macrophages can occur via membrane-bound and intracellular pattern recognition receptors (PRRs), which are expressed in macrophages as well as in vascular cells [12]. Using gene deficient mice, a pro-atherosclerotic role was found for MyD88, a key adaptor protein in the signaling pathways of most Toll-Like Receptors (TLRs) [13] and of interleukin (IL)-1 $\beta$  and IL-18, two pro-atherosclerotic cytokines [14]. By binding to membrane-bound CD36 and TLR4/6 on macrophages, oxLDL induces cytokine and chemokine production via NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation [15]. In addition, modified LDL can induce a pro-inflammatory response in macrophages through

TLR2 and TLR4 [16]. Furthermore, not only extracellular, but also intracellular PRRs are known to be involved in atherosclerotic inflammation. Minute cholesterol crystals have been reported to induce inflammation in macrophages by stimulating the caspase-1-activating NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome with subsequent IL-1 $\beta$  and IL-18 activation [17]. In advanced atherosclerotic lesions, macrophages contribute to changes in plaque morphology, including formation of a necrotic core and thinning of the overlying fibrous cap, ultimately leading to ‘vulnerable plaques’, which are prone for rupture [18]. For a more detailed overview of the role of monocytes/macrophages in the process of atherosclerosis, we refer to recent excellent reviews on this topic [2, 18, 19].

### The concept of ‘Trained immunity’

Recently, the classic dichotomy that only adaptive immune cells and not innate immune cells have an immunological memory was challenged [3]. It has long been known that plants and invertebrates, which lack adaptive immunity, are able to build up an immunological memory through chromatin modification in cells of their innate immune system [20]. Already many years ago, bacille Calmette- Guérin (BCG) vaccine was shown to protect mice not only against mycobacteria but also against unrelated pathogens [21, 22]. Along the same line, BCG vaccination in children in West Africa was found to decrease morbidity due to infections other than tuberculosis [3, 23]. Based on these observations, a memory for cells of the innate immune system was suspected, and this has been termed as ‘trained immunity’[3]. In a series of recent experiments, we were able to provide a solid basis for the concept of trained immunity, as our group demonstrated it in vivo in animals lacking T and B lymphocytes, and in humans, as well as in vitro with isolated monocytes [4, 5].

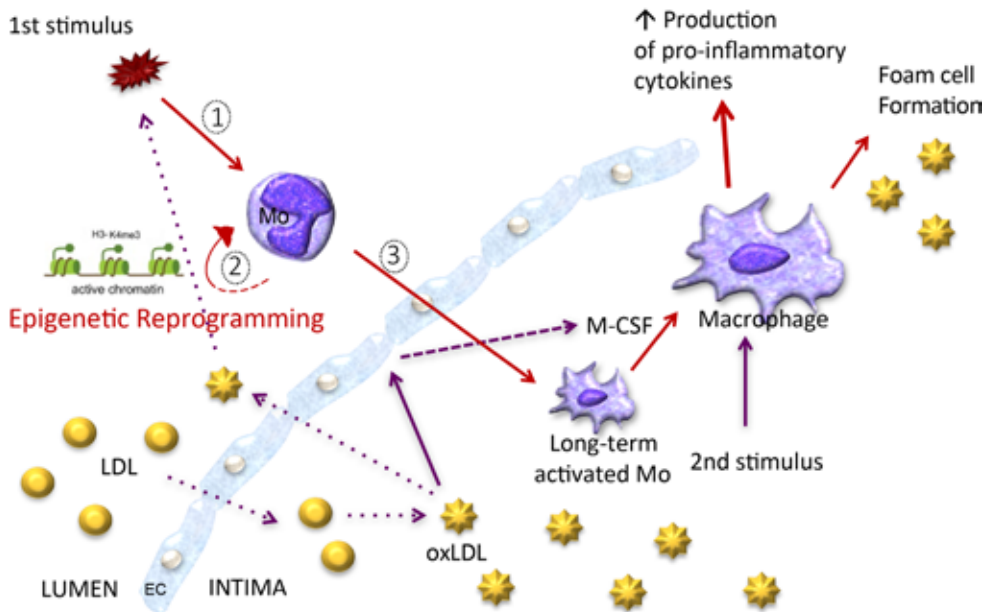
In healthy humans, BCG vaccination induces an augmented monocyte production of pro-inflammatory cytokines, such as TNF $\alpha$  and IL-6, when exposed to unrelated microbial products [4]. This enhanced pro-inflammatory state of monocytes could be detected even beyond 6 months after vaccination. In mice that are genetically deficient of T- and B-lymphocytes, BCG vaccination was able to protect mice against disseminated candidiasis upon reinfection, indicating a memory independent of the adaptive arm of the immune system. In addition to BCG, ‘trained immunity’ also can be induced by *Candida albicans*: Quintin et al. showed that infection with *C. albicans* induced a similar reprogramming of monocytes as seen with BCG vaccination, resulting in an enhanced cytokine production in vivo and in vitro upon re-stimulation with various agents (e.g. TLR2 and TLR4 agonists or unrelated bacterial components) [5].

In these studies, it has also been demonstrated that trained immunity induced by either BCG, *C. albicans* or the fungal cell wall component  $\beta$ -glucan, is mediated via epigenetic reprogramming. Epigenetic regulation refers to the modification of chromatin, resulting in differential regulation of gene expression, but without modification of the nucleotide sequence of the DNA itself [24]. Currently, there are three different epigenetic modifications known: DNA-based modifications, e.g. DNA methylation or hydroxymethylation, which silences the gene transcription; RNA-based modifications, which can either repress or activate gene transcription and thirdly, posttranslational modifications of histones [25-27]. In the cell nucleus, the DNA is wrapped around an octamer of histones: 2 dimers of Histone 2A (H2A) and Histone 2B (H2B) and 2 dimers of H3 and H4 [28]. These histones are the primary protein content of the chromatin, packaging and ordering the DNA into nucleosomes. They can undergo posttranslational modification on the N-terminal tails ("epigenetic modification"), by methylation, acetylation and phosphorylation. Modification alters their interaction with the DNA and thereby facilitates or represses DNA transcription, depending on the type of modification [29]. Acetylation is often associated with transcriptional activation, but histone methylation is either repressing or activating, depending on the methylation residue and the amount of methyl-groups added, e.g. mono-, di- or tri-methylation [25]. For instance, H3K27 trimethylation is a repressive mark, whereas H3K4 mono- or trimethylation is a transcriptionally activating mark.

The pro-inflammatory phenotype of monocytes in BCG vaccinated subjects was shown to be associated with an increased trimethylation on histone 3 at lysine 4 (H3K4me3), that is linked with enhanced mRNA transcription [29]. Similarly, monocyte training by *C. albicans* induced a genome-wide H3K4 trimethylation profile, suggesting involvement of the chromatin modifications as substrate for trained immunity, similarly to the innate memory described in plants [20]. Upon *C. albicans* or  $\beta$ -glucan training, more than 5000 genes displayed increased trimethylation at the H3K4 and these genes could be clustered mainly into genes involved in immune defense, such as cytokines, C-type lectin receptors and TLRs. Interestingly, also structural, metabolic and signaling pathway genes had upregulated H3K4me3.

## Can trained immunity contribute to atherogenesis?

Monocytes/macrophages play a central role in the development of atherosclerosis. As microbial products can induce long-lasting epigenetic reprogramming of monocytes, leading to an augmented pro-inflammatory response, it is logical to hypothesize that these mechanisms may be relevant for atherogenesis. Stimulation of long-term activated macrophages by inflammatory stimuli within the vascular wall, including agonists of TLR2 and



**Figure 1:** Schematic representation of the concept that epigenetic reprogramming of monocytes/macrophages is involved in the initiation or progression of atherosclerosis. Initial stimulation of monocytes by microorganisms or alternative stimuli induces a long-term activated phenotype via epigenetic reprogramming. This phenotype facilitates monocyte recruitment and subsequent atherogenesis. EC, endothelial cell; M-CSF, monocyte-colony stimulating factor; Mo, monocytes

TLR4, might result in an exaggerated inflammatory response, thereby promoting the induction or amplification of atherosclerosis (Fig. 1).

It has been previously described that epigenetic modifications can be long-lasting after an experimental model of infection in mice [30] or after BCG vaccination in humans [4]. Indeed, BCG vaccination exerts a nonspecific protection against infections throughout childhood [31]. If the epigenetic reprogramming of monocytes appears to be long-lived and play a role in atherosclerosis, we might have to redesign therapeutics. What is the available evidence to support our hypothesis?

Already in 1999, BCG vaccination has been associated with an exaggerated atherosclerosis. Lamb et al. reported that administration of BCG in rabbits fed a cholesterol-enriched diet augments adherence of mononuclear cells to aorta endothelium [32], attenuates endothelium-dependent vasodilation [33], increases aortic intima/media thickness, and increases aortic atherosclerotic lesion formation [34]. Also, increased peripheral lymphocyte and monocyte activation was shown by enhanced CD11b and CD25 expression [32]. At that time, these observations were suggested to be due to BCG-induced antibodies against heat-

shock protein 65, which can also target vascular HSP-60, thus leading to vascular injury [32-34]. Obviously, a role for the adaptive immune system (like antibody formation) cannot be excluded in these experiments, but it is tempting to primarily explain the findings by BCG-induced epigenetic reprogramming of monocytes. In addition, Cinemre et al. reported a positive association between the titer of anti-Saccharomyces cerevisiae antibodies (ASCA), which can be induced by *C. albicans* [35] and the occurrence of an acute myocardial infarction [36]. Furthermore, Nagi-Miura et al. described the induction of lethal and severe coronary arteritis by a pathogen-associated molecular pattern (PAMP) produced by *C. albicans* [37]. Here too, the exquisite ability of *Candida* components to induce a trained state of monocytes could well explain these observations.

In addition to the above-mentioned data, trained immunity might explain the association between infections and atherosclerosis that have been reported in the literature. A variety of microorganisms have been linked to an increased risk of cardiovascular disease [38]. Although surrounded by quite some controversy, many studies suggest that *C. pneumoniae* promotes atherogenesis: upon exposure to *C. pneumoniae*, isolated endothelial cells demonstrate an enhanced expression of adhesion molecules [39]; *C. pneumoniae* stimulates cytokine production by monocytes [40] and induces foam cell formation [41], and *C. pneumoniae* induces human vascular smooth muscle cells to proliferate and release prototypical atherogenic cytokines [42]. In rabbits and mice, infection with *C. pneumoniae* can initiate atherosclerosis [43]. These preclinical findings have been supported by observational studies in humans, which report an association between the burden of prior infections (including *C. pneumoniae*, *H. pylori*, cytomegalovirus, and herpes simplex viruses) and carotid plaque thickness, cardiovascular events, and stroke [44-46]. However, the strength of these data associating pathogens with atherosclerosis varies considerably and up till now, the mechanism underlying this association is unclear [47]. Theoretically, training of monocytes by these microorganisms could be an underlying mechanism linking infection to atherogenesis. To take this one step further, the trained immunity concept does not require that the causative agent remains present in the host. Some organisms might reprogram the monocyte by a hit-and-run mechanism.

It is also important to realize that induction of trained immunity does not have to be restricted to microbial products. As described earlier, oxLDL and cholesterol crystals are able to induce an inflammatory response in monocytes by stimulation of various PRRs, and therefore they might also have the potential to epigenetically reprogram monocytes.

Interestingly, in the past decade, several epigenetic marks such as DNA methylation and

histone acetylation have been associated with atherosclerosis [25, 48]. DNA methylation is by far the most studied phenomenon and global DNA hypermethylation has been reported in atherosclerotic patients [49-51]. However, this would result in decreased gene transcription and could not explain the primed status of a trained immune cell. Furthermore, histone modifications, in particular histone acetylation, have only been studied in vascular smooth muscle cells and endothelial cells in the context of atherosclerosis. N'Guessan et al. showed that oxLDL increased global H4 acetylation in isolated endothelial cells resulting in upregulated interleukin-8 and Monocyte Chemoattractant Protein-1 production [52]. Live *C. pneumoniae* also induced H4 acetylation in isolated ECs in vitro resulting in upregulation of cytokine production [53]. Furthermore, the transcription of the nitric oxide synthase (NOS)3 gene, encoding eNOS, is activated in endothelial cells and repressed in vascular smooth muscle cells via several epigenetic marks [54]. Environmental stimuli such as hypoxia [55] or reduced shear stress [56, 57] reduce the acetylation but also H3K4 methylation on this gene in endothelial cells, underlining the importance of histone modifications in the environmental risk factors for cardiovascular disease.

### New therapeutic approaches

Epigenetic modifications are known to act as mediators between the environment and the genome, leading to modification of gene transcription. Since these are dynamic and reversible changes, they become interesting targets for new therapeutics [58-60]. Histone methylation processes can be divided into arginine methylation and lysine methylation, as well as in mono-, di- and tri-methylation. Interfering with this complex balance might be difficult, since the various forms of methylation differ in their effects on transcriptional activity. Only recent efforts have begun to focus on the pharmacological modulation of specific histone methyltransferases and demethylases and current knowledge is reviewed in [61]. The concept that pharmacological modulation of histone modifications can affect the process of atherosclerosis has been tested in murine models of atherosclerosis: the histone deacetylase (HDAC) inhibitor trichostatin A appeared to exacerbate atherosclerosis in LDL receptor<sup>-/-</sup> mice [62].

Since it is well known that epigenetic changes contribute to the development of cancer [63], it is not surprising that various small molecules that reverse epigenetic (in)activation are already undergoing clinical trials. Promising histone methyltransferase inhibitors studied are S-adenosylmethionine (SAdMe) and its metabolite methyl thioadenosine. The latter reduced inflammation-induced colonic cancer and inhibited several pathways important in carcinogenesis [64]. Histone methylation is also known to be involved in immunological responses, as it regulates antigen-driven T-cell response and genes important in survival,

proliferation and differentiation [65, 66]. Inhibition of histone methylation by DZNep was shown to arrest ongoing graft-versus-host disease (GVHD) in mice after allogeneic bone marrow transplantation. Besides DZNep, BIX-01294 is also known to be a specific inhibitor of lysine methyltransferases [67].

## Conclusion

In conclusion, the studies summarized above demonstrate that epigenetic changes at the histone level are amenable to pharmacological modulation. Further elucidation of the role of epigenetic reprogramming of monocytes in atherogenesis is likely to provide novel targets to combat the development and progression of atherosclerosis.

Considering the breadth of evidence in the literature linking epigenetic reprogramming of innate immune cells with long-term inflammatory profiles on the one hand, and inflammation and epigenetic changes with atherosclerosis on the other hand, it is rational to hypothesize that the concept of trained immunity has an important role in atherosclerosis. This concept could explain some of the current controversies concerning the variable association of pathogens with atherosclerosis. Studying the role of trained immunity in atherosclerosis might give insight in the disease mechanism and has great potential to reveal new drug targets.



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PART I

TRAINED IMMUNITY AND  
ATHEROSCLEROSIS IN VITRO



# CHAPTER 3

## IN VITRO EXPERIMENTAL MODEL OF TRAINED INNATE IMMUNITY IN HUMAN PRIMARY MONOCYTES

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## ABSTRACT

### Background

Innate immune memory or 'trained immunity' has been recently described as an important property of cells of the innate immune system. Due to the increased interest in this important new field of immunological investigation we sought to determine the optimal conditions for an in-vitro experimental protocol of monocyte training using three of the most commonly used training stimuli from the literature:  $\beta$ -glucan, the Bacille Calmette-Guerin (BCG) vaccine, and oxidized LDL (oxLDL).

### Methods

We investigated and optimized a protocol of monocyte trained immunity induced by an initial training period with  $\beta$ -glucan, BCG or oxLDL, followed by washing and resting of the cells, and thereafter restimulation with secondary bacterial stimuli. The training and resting time intervals varied to identify the optimal setting for long-term induction of trained immunity. Trained immunity was assessed in terms of secondary cytokine response, production of reactive oxygen species, cell morphology and induction of glycolysis.

### Results

Monocytes primed with  $\beta$ -glucan, BCG and oxLDL showed increased pro- and anti-inflammatory cytokine responses upon restimulation with non-related stimuli. Also, all three stimuli induced a switch to glycolysis (Warburg effect). These effects were most pronounced when training interval was 24h training and resting time interval was 6 days. BCG and oxLDL training also led to increased production of reactive oxygen species, whereas  $\beta$ -glucan training showed a decrease in reactive oxygen species production.

### Conclusion

We describe the optimal conditions for an in vitro experimental model in human primary monocytes to study the induction of trained innate immunity by microbial and metabolic stimuli.

## INTRODUCTION

The immune response is a complex system of cellular and humoral components, which have the ability to detect 'non-self' structures and confer protection against invading pathogens, playing a crucial role in survival of multicellular organisms. Traditionally the immune response has been divided into the innate immune system and the adaptive immune system. Adaptive immunity, with T- and B-cells as cellular effectors, develops over several weeks after birth, is highly specific and builds a specific immunological memory, leading to protection against reinfection. The innate immune system on the other hand is classically thought to act rapidly in a non-specific and identical manner every time it encounters a pathogen, without having the ability to build an immunological memory.

Recently, the concept that innate immunity is non-specific and completely lacks immunological memory has been challenged. First, the discovery of Pattern Recognition Receptors (PRRs) confers some specificity to innate immunity and secondly, a growing body of literature shows that innate immunity can adapt its function after a first insult (1, 2). Several studies have shown that plants and invertebrates, organisms which lack an adaptive immune system, show enhanced immune responses upon reinfection (3). This phenomenon has recently been confirmed in higher vertebrates and humans, and has been named 'trained immunity' or 'innate immune memory'. Trained immunity is dependent on epigenetic remodelling and on rewiring of intracellular metabolic pathways, which in turn lead to a long-term pro-inflammatory phenotype that is characterized by increased cytokine responses (4, 5).

A series of recent in vitro and in vivo experiments have revealed that the phenomenon of trained immunity is induced both by Pathogen Associated Molecular Patterns (PAMPs) and a number of Danger Associated Molecular Patterns (DAMPs). First, in mice, a single dose of *C. albicans* confers protection against reinfection with *S. aureus*. In vitro studies showed that the *C. albicans* cell wall component  $\beta$ -glucan induces epigenetic remodelling and functional reprogramming through a dectin-1/Raf1-dependent pathway (6, 7). Second, BCG vaccination of healthy human volunteers resulted in non-specific upregulation of ex vivo cytokine production of isolated monocytes that persisted for at least 3 months after vaccination. This effect is dependent on NOD2 signalling, autophagy and epigenetic changes in histone methylation leading to increased transcription, and likely explains, at least in part, the observed non-specific effects of BCG on overall mortality (8–10). Thirdly, the atherogenic lipid oxidized LDL (oxLDL) induces long-term pro-atherogenic changes in monocytes in vitro leading to increased pro-atherogenic cytokine expression, increased scavenger receptor expression and decreased cholesterol efflux transporter expression and ultimately in increased foam cell formation. This effect is dependent on TLR4/TLR2 and subsequent PI3K and ERK activation, epigenetic changes in histone methylation (e.g. H3K4me3, H3K9me3 and

H3K27me3) resulting in increased transcription (11, 12).

In recent years, there is an increased interest in immunological research to the study of trained immunity, with studies assessing different organisms and diseases (13–17). Therefore, we sought to determine the optimal conditions for an in-vitro experimental protocol of monocyte training using three of the most commonly used training stimuli from the literature:  $\beta$ -glucan, BCG vaccine, and oxLDL.

## METHODS

### Reagents

$\beta$ -1,3-(D)-glucan was kindly provided by Professor David Williams (TN, USA), Bacille Calmette-Guérin (BCG vaccine SSI) was obtained from the Netherlands Vaccine Institute. Oxidized LDL (oxLDL) was prepared as described before (18). *Escherichia coli* LPS (serotype 055:B5, Sigma-Aldrich) was further purified as described (19), Pam3Cys was obtained from EMC microcollections (L2000). Luminol and zymosan (from *S. cerevisiae*) were purchased from Sigma-Aldrich.

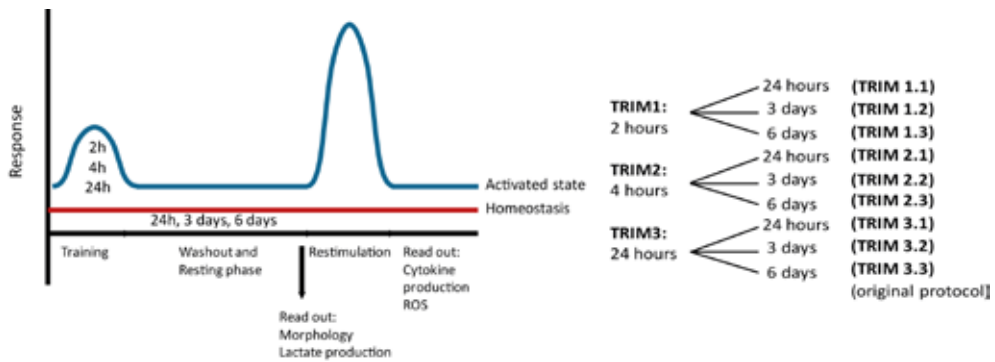
### PBMC and monocyte isolation

Buffy coats from healthy donors were obtained after written informed consent (Sanquin blood bank, Nijmegen, The Netherlands). PBMC isolation was performed by dilution of blood in sterile PBS and density centrifugation over Ficoll-Paque (GE healthcare, UK). Cells were washed three times in cold PBS. Percoll isolation of monocytes was performed as described (20). Briefly,  $150\text{--}200 \cdot 10^6$  PBMCs were layered on top of a hyper-osmotic Percoll solution (48,5% Percoll (Sigma-Aldrich, St Louis, MO, USA), 41,5% sterile  $\text{H}_2\text{O}$ , 0.16M filter sterilized NaCl) and centrifuged for 15 minutes at 580xg. The interphase layer was isolated and cells were washed with cold PBS. Cells were resuspended in dutch modified RPMI culture medium (Roswell Park Memorial Institute medium; Invitrogen, CA, USA) supplemented with 10 $\mu\text{g}/\text{ml}$  gentamicin, 10mM Glutamax, and 10mM pyruvate and counted. An extra purification step was added by adhering Percoll isolated monocytes to polystyrene flat bottom plates (Corning, NY, USA) for 1 hour at 37°C; the cells were washed with warm PBS to obtain maximal purity. At this point T-cell contamination as measured by FACS was 5% (Supplementary Figure 1).

### Trained immunity model in human monocytes

100.000 cells/well were added to flat bottom 96-well plates. After incubation for 1 hour at 37°C and washing with warm PBS, monocytes were incubated with culture medium only as a negative control or 5  $\mu\text{g}/\text{ml}$   $\beta$ -glucan, 10  $\mu\text{g}/\text{ml}$  BCG or 10  $\mu\text{g}/\text{ml}$  oxLDL for 2, 4 or 24h (in 200  $\mu\text{L}/\text{well}$  RPMI + 10% pooled human serum) (Figure 1). After the indicated incubation time, the cells were washed once with 200 $\mu\text{L}$  warm PBS and incubated for 24h, 3 or 6 days in culture medium with 10% serum and medium was changed once at day 3 for cells resting 6 days. Cells were re-stimulated with 200 $\mu\text{L}$  RPMI, 10 ng/ml LPS, or 10 $\mu\text{g}/\text{ml}$  Pam3Cys. After 24h, supernatants were collected and stored at -20°C until cytokine measurement. Supernatants before re-stimulation were stored at -20°C for lactate measurements.

For production of reactive oxygen species (ROS) and for cell counting experiments,  $5 \times 10^6$



**Figure 1:** Schematic overview of trained immunity methodology. Monocytes were trained for 2 hours (TRIM1), 4 hours (TRIM2) or 24 hours (TRIM3). After washing away the training stimulus, cells were rested for 24 hours (TRIM#.1), 3 days (TRIM#.2) or 6 days (TRIM#.3), after which the cells were restimulated with RPMI, LPS or Pam3Cys for 24 hours.

monocytes were trained in vitro in 10cm petridishes (Greiner) in 5 ml medium volumes for 24h as described above with a 6 day resting period. At day 6, cells were detached from the plate with versene (Life Technologies) and counted. 25.000 cells/well were restimulated as described above. 100.000 cells/well were used for ROS measurement, as described below.

### Microscopy

Cell morphology was studied by conventional light microscopy during incubation each day. Pictures were taken before restimulation at 4x and 20x magnification. Cell size was quantified using Leica LAS EZ software (Leica Microsystems).

### Reactive oxygen species measurements

For measurement of reactive oxygen species production a luminol – enhanced luminescence assay was used. After detachment and counting of trained monocytes, a total of  $1 \times 10^5$  cells were added per well of a white 96-well assay plate (Corning) in a volume of 200  $\mu$ l. Cells were left either unstimulated or stimulated with serum opsonised zymosan in a concentration of 1 mg/ml. Luminol (145  $\mu$ g/ml) was added and chemiluminescence was measured every 142 s for 1h. All measurements were performed at least in duplicate depending on the number of cells available. Opsonized zymosan particles were prepared by incubation of zymosan derived from *Saccharomyces cerevisiae* (Sigma, St. Louis) in pooled human serum for 30 minutes at 37°C, after which particles were washed twice in PBS and suspended in PBS.

### Cytokine and lactate measurements

Cytokine production was determined in supernatants using commercial ELISA kits for TNF- $\alpha$ , IL-1Ra (R&D systems, MN, USA) and IL-6 and IL-10 (Sanquin, Amsterdam, The Netherlands)

following instructions of the manufacturer. Lactate concentration was measured using a Lactate Fluorometric Assay Kit (Biovision, CA, USA).

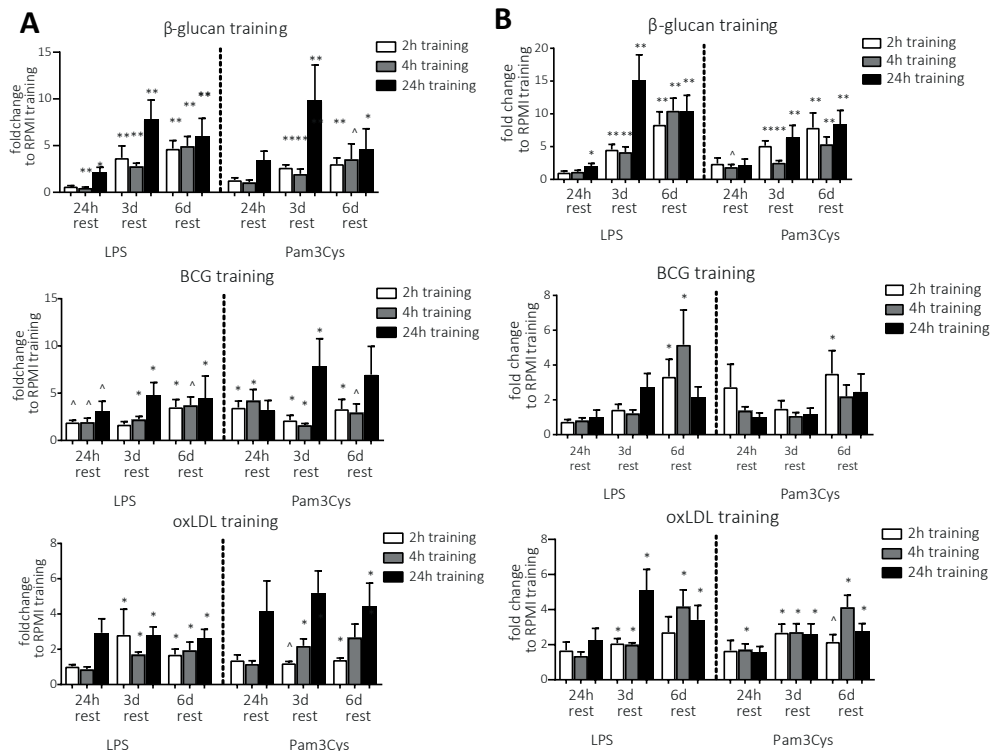
### Statistics

In vitro monocyte experiments were performed at least 6 times and analysed using a Wilcoxon matched pairs signed-rank test, comparing fold change or raw cytokine values to the RPMI control. A two-sided P-value below 0.05 was considered statistically significant. All data were analyzed using Graphpad prism 5.0 (La Jolla, CA, USA).  $^{\wedge}$  =  $p = 0.06$ , \* =  $p < 0.05$ , \*\* =  $p < 0.01$ . Data are shown as means  $\pm$  SEM.

## RESULTS

### Training induces an increase in pro-inflammatory cytokine production, which is dependent on both training and resting time

As was shown previously, 24 hour incubation with  $\beta$ -glucan, BCG, or oxLDL induces a trained immune phenotype characterized by increased production of pro-inflammatory cytokines upon restimulation at day 7 with heterologous stimuli. Here we aimed to investigate how temporal changes in duration of the first stimulus and the timing of the second stimulus impact upon the development of the trained immunity phenotype. (Figure 2A-B and Supplementary Figure 2). Training with  $\beta$ -glucan induced the strongest increase in cytokine production upon restimulation, whereas the effects of BCG and oxLDL were less pronounced, although still approximately associated with a 4-5 fold higher production of cytokines. For  $\beta$ -glucan and oxLDL training, the fold increase of IL-6 and TNF $\alpha$  production was comparable, while priming with BCG led to a greater amplification of IL-6 (Fig 2A) compared to TNF $\alpha$  (Fig 2B) production.

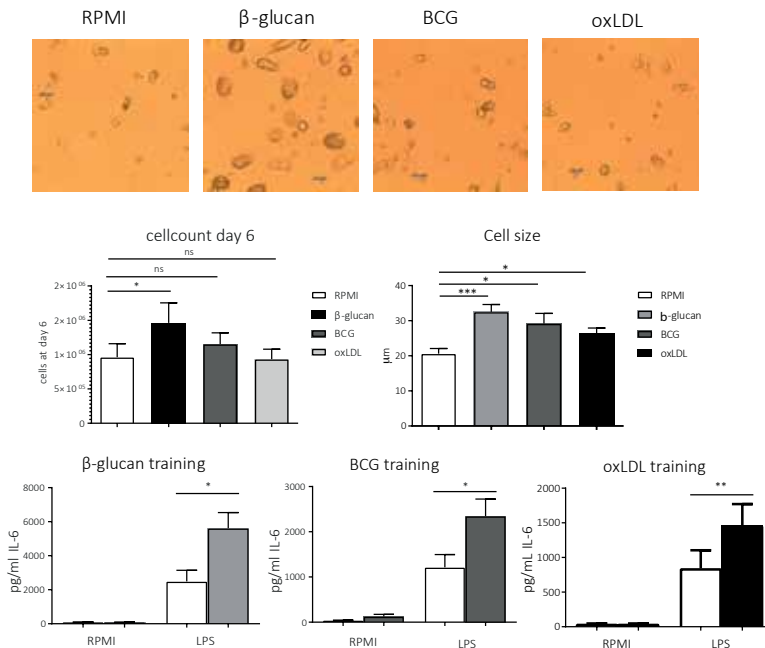


**Figure 2:** Increased pro-inflammatory cytokine production is dependent on both training interval and resting time. (A) IL-6 production after re-stimulation; cells were trained for 2h, 4h or 24h with  $\beta$ glucan (upper panel), BCG (middle panel) or oxLDL (lower panel), and rested for 24h, 3d or 6d. (B) TNF $\alpha$  production after re-stimulation; cells were trained for 2h, 4h or 24h with  $\beta$ glucan (upper panel), BCG (middle panel) or oxLDL (lower panel), and rested for 24h, 3d or 6d. Shown are fold changes compared to the RPMI control (n=6, ^p=0.06, \*p<0.05, \*\*p<0.01 compared to RPMI control).

For all three stimuli, priming for either 2 hours, 4 hours or 24 hours induced training, when the cells were rested for three or six days. However, the fold increase in cytokine production was highest with 24h training time interval. When comparing the different time periods between stimulation and restimulation, it follows that the trained phenotype developed only after at least three days, with a maximum response after 6 days.

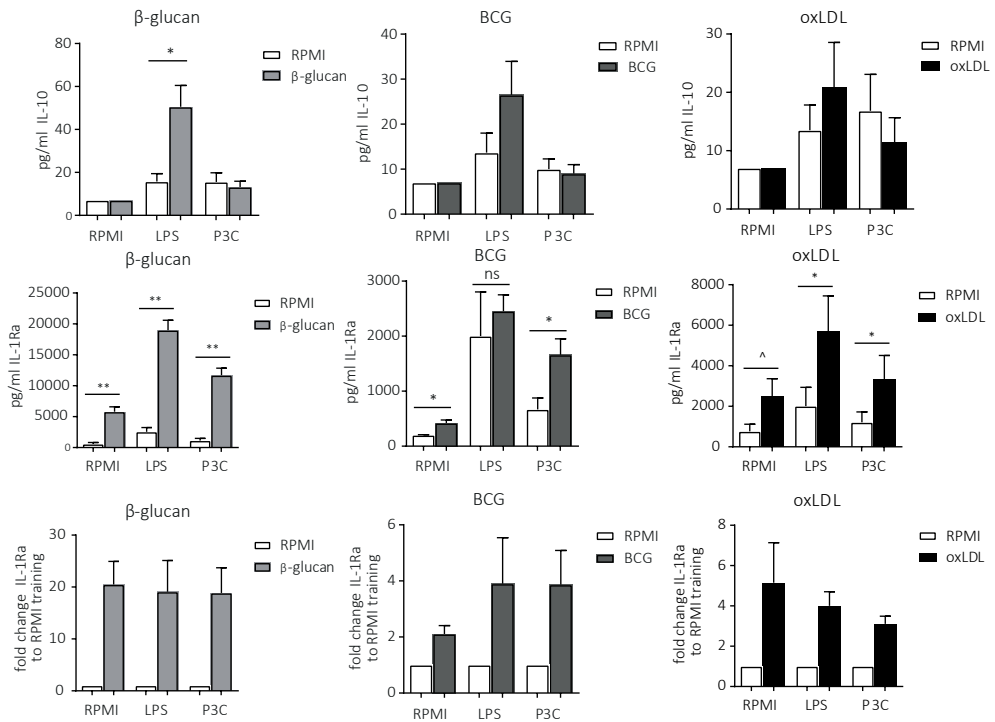
### Training associated morphological changes

Upon training, the monocytes not only changed their response to secondary stimuli and subsequently their cytokine production, but also changed their morphology. Just before restimulation, we analyzed cell morphology and cell size by using light microscopy at two different magnifications (Fig 3A and supplementary figure 3). When cells were trained for 24 hours and rested for 6 days,  $\beta$ -glucan induced most remarkable changes in cell morphology: the cells were bigger than non-trained cells. BCG and oxLDL training only induced minor changes in cell morphology, with a slight increase in cell-size compared to the untrained control cells (Fig 3B). We also analyzed cell numbers at the end of the most optimal protocol of trained immunity induction (24h training, 6 days rest) by culturing cells in Petri dishes, detaching them on day 6 before restimulation, and reseeding corrected cell numbers for each



**Figure 3:** Trained immunity effects on cell morphology and numbers. (A) Cell morphology of cells from TRIM3.3 trained with RPMI (negative control)  $\beta$ glucan, BCG or oxLDL. Pictures were taken before re-stimulation at day 6 (20x). (B) Relative cell counts before re-stimulation at day 6  $n=6$ , \*  $p<0.05$ , compared to RPMI control). (C) IL-6 and TNF $\alpha$  production after re-stimulation for corrected cell counts. ( $n=6$ , \*  $p<0.05$ , \*\*  $p<0.01$  compared to RPMI control).





**Figure 4:** Anti-inflammatory cytokine production is increased in trained monocytes. (A) IL-10 production after re-stimulation; cells were trained for 24h with  $\beta$ glucan, BCG or oxLDL and rested for 6d. Production of the anti-inflammatory cytokine IL-10 increases upon training. (B). IL-1Ra production; cells were trained for 24h with  $\beta$ glucan, BCG or oxLDL and rested for 6d. Production of the anti-inflammatory cytokine IL-1Ra increases upon training, not only after restimulation, but also on baseline. (n=6, ^p=0.06, \* p<0.05, \*\* p<0.01 compared to RPMI control).

condition. Counting experiments showed that  $\beta$ -glucan training induced a small increase in cell numbers compared to the RPMI control (Figure 3C), whereas BCG or oxLDL training did not induce this increase in numbers. However, after correcting for cell numbers, the cytokine production upon restimulation was still significantly increased, indicating that the increase in cytokines was not due to an increased cell number, but is mainly dependent on the production of cytokines per cell (Figure 3D).

### The production of anti-inflammatory cytokines IL-10 and IL-1Ra is increased in trained cells

Previously we have shown that  $\beta$ -glucan training increases not only pro-inflammatory cytokine production, but also anti-inflammatory cytokine production. Therefore, we measured the production of two anti-inflammatory cytokines IL-10 and IL-1Ra upon restimulation for all three training stimuli and all training protocols. Training was associated with increased LPS-induced IL-10 production depending on training time interval and resting time (Figure 4A

and Supplementary Figure 3A), but this only reached statistical significance for training with  $\beta$ -glucan. The production of IL-1Ra was increased for all three training stimuli even without re-stimulation (Figure 4B), and this was dependent on training duration and resting time (Supplementary Figure 3B).

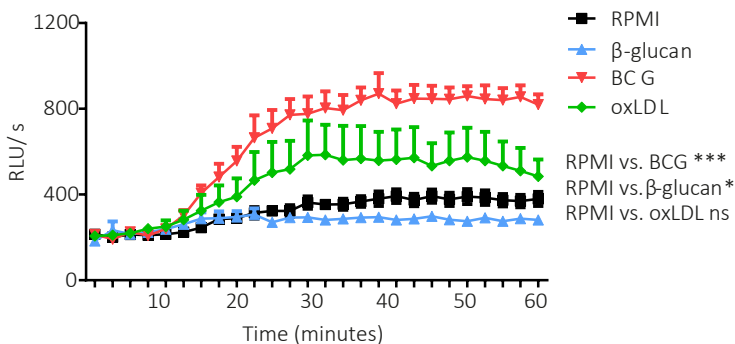
### ROS production is increased upon BCG and oxLDL training, but not upon $\beta$ -glucan training

To further characterize the functional phenotype of trained monocytes we measured another innate immune effector mechanism, the oxidative burst, in the most optimal protocol (24h training, 6 days rest).

Both BCG and oxLDL trained cells showed increased production of reactive oxygen species (ROS) compared to RPMI control when cells were stimulated with zymosan, although for oxLDL this effect was not statistically significant. Interestingly,  $\beta$ -glucan trained cells showed a very different pattern, with a slight, but statistically significant downregulation of ROS production.

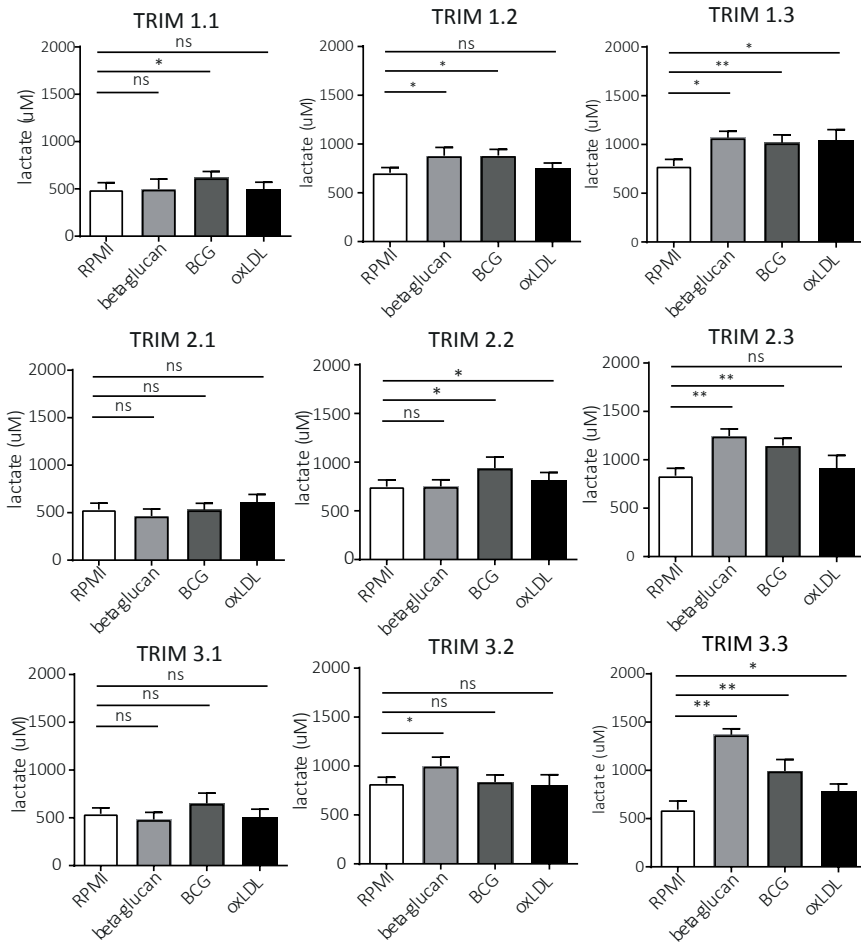
### The metabolic switch towards increased glycolysis induced upon training is dependent on both training and resting time

Previously, we have shown that trained immunity depends on a metabolic shift from oxidative phosphorylation towards aerobic glycolysis (Warburg effect) (21). Increased glycolysis results in increased lactate production by the cell, which can be easily be measured by analyzing the accumulation of lactate in the culture medium. Before the cells were restimulated, the culture medium was removed and stored and lactate concentration was measured (Fig 6). 24h resting time, independent of the training time, did not increase glycolysis and lactate production. When cells were rested for 3 days, lactate production was slightly increased for 2 hour and 24 hour  $\beta$ -glucan trained cells; for BCG training, 2h and 4h training induced a



**Figure 5:** ROS production is a component of trained immunity for some stimuli. ROS production is significantly increased for BCG and oxLDL trained monocytes;  $\beta$ -glucan training decreases ROS production. ( $n=6$ , \*  $p<0.05$ , \*\*\*  $p<0.001$  compared to RPMI control)..

slight increase in glycolysis, while for oxLDL training only 4h training period was sufficient to induce significant increase in lactate production. When cells were rested for 6 days however, all three training durations induced significant increase in lactate production for all three stimuli. Interestingly, the lactate production and thus glycolysis was even more increased with increasing training duration.



**Figure 6:** Trained immunity induction of glycolysis. Cells were trained for 2h, 4h or 24h with RPMI (white, negative control)  $\beta$ glucan (light grey), BCG (dark grey) or oxLDL (black), and rested for 24h, 3d or 6d. Lactate production was measured in the supernatants before re-stimulation. (n=6, \*  $p < 0.05$ , \*\*  $p < 0.01$  compared to RPMI control).

## DISCUSSION

In the present study we describe the optimal conditions for the induction of trained immunity in an in vitro model in primary human monocytes. This model has previously been used to investigate several different stimuli, but the optimal parameters necessary to induce trained immunity have not been yet characterized. In this model, microbial or endogenous stimuli such as  $\beta$ -glucan, BCG, oxidized LDL are added to monocytes for a short period of time (training period), after which cells are washed and let to rest in culture medium, followed by restimulation with unrelated antigens (6, 8, 11, 22, 23).

This study provides several new insights in this basic model of trained innate immunity, that will aid the design of future studies. Firstly, induction of trained immunity depends on the duration of the first stimulation time interval: the ‘training period’, with increasing training time leading to increased induction of trained immunity, an effect which was observed for all three stimuli. Secondly, the magnitude of the induction of non-related cytokine responses increases with the time that trained cells are left to rest, most notably for  $\beta$ -glucan primed cells. The importance of resting time to establish trained immunity has recently also been demonstrated in a study which described the role of the transcription factor ATF7 in mediating epigenetic changes involved in innate immune memory, most notably after LPS stimulation. Three days after the first LPS challenge, the expression of TNF $\alpha$  and Cxcl2 was decreased upon rechallenge with LPS, whereas the opposite was true three weeks after LPS challenge. Multiple pro-inflammatory genes were upregulated at the later time point, which was accompanied by reduced epigenetic marks associated with gene silencing and incomplete recruitment of ATF7, which is associated with formation of heterochromatin. This study also found that treatment with  $\beta$ -glucan induced phosphorylation of ATF7 analogous to treatment with LPS (24). Similarly, our study shows induction of tolerance, most notably with  $\beta$ -glucan and BCG, when cells are only briefly left to rest before rechallenge (24 hours), whereas a training effect is seen if cells are left to rest for 3 or 6 days. This could suggest the intriguing possibility that ATF7 or analogous mechanisms may be at play here. The importance of resting time in the induction of trained immunity is also shown by the remarkable increases in glycolysis of trained cells after 6 days of rest, compared to 1 day or 3 days of resting time.

Another insight from this study is that trained immunity does not translate only into increased pro-inflammatory response of monocytes, but is likely to involve a more general increase in the responsiveness of the cells, as witnessed by the amplification of both pro- and anti-inflammatory cytokines production, ROS production (in case of BCG and oxLDL) and metabolic activation. We should be of course cautious in the extrapolation of all the details

of the in-vitro model to the in-vivo situations: for example vaccination trials performed both in infants and in adults showed increased pro-inflammatory cytokine responses after BCG vaccination, whereas no effects were noted on anti-inflammatory cytokine responses (8, 23, 25). Furthermore, in patients suffering from elevated lipoprotein(a), which induces trained innate immunity in vitro, the anti-inflammatory cytokine response was even decreased (Van der Valk et al., in press).

It is also important to note that different inducers of trained immunity lead to different phenotypical and functional changes, as witnessed by light microscopy, cytokine production and induction of ROS. Although a common denominator for the three priming stimuli studied here is the upregulation of both pro- and anti-inflammatory cytokine responses and the stimulation of glycolysis, other physiological processes such as induction of ROS or cell proliferation differ between different priming stimuli. When cytokine responses were corrected for cell counts, the cytokine responses were still strongly upregulated nevertheless. All in all however, these data suggest that each of these training stimuli induces different trained immunity functional programs, and this is not a surprise considering their diverse nature (5).

In conclusion, although the effects of these three stimuli on trained immunity have been described before, this is the first time that the kinetics of this in vitro model have been studied. This study describes the characteristics of an in vitro model to study the influence of different stimuli on the induction of trained innate immunity. This is a practical and useful methodological tool that will assist the research community in the investigation of this emerging field of immunology.

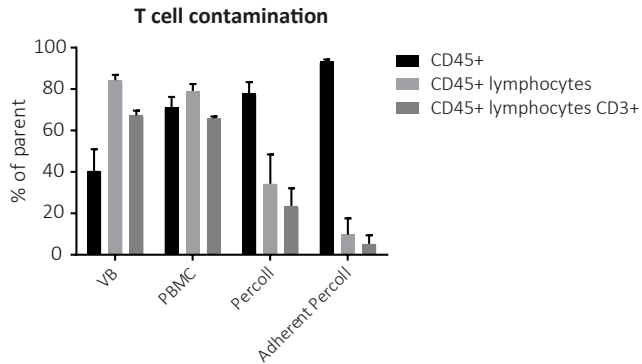
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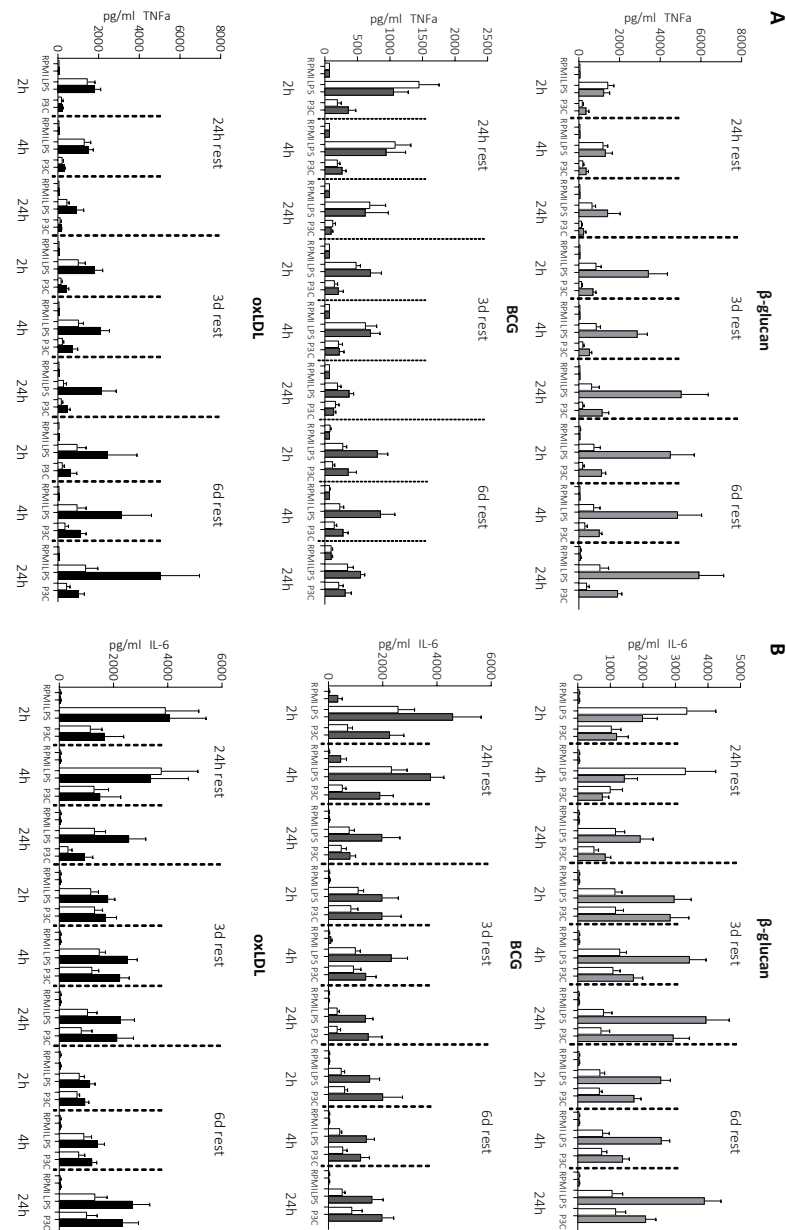
## SUPPLEMENTARY FIGURES

**Supplementary Figure 1.** Monocyte purity of different isolation steps. The proportion of T-cells was determined in whole blood, the PBMC fraction after Ficoll density gradient, the purified monocyte fraction after Percoll density gradient and after 1 hour adherence and washing of the purified Percoll isolated monocytes. T-cell contamination is approximately 5% after adherence and washing.

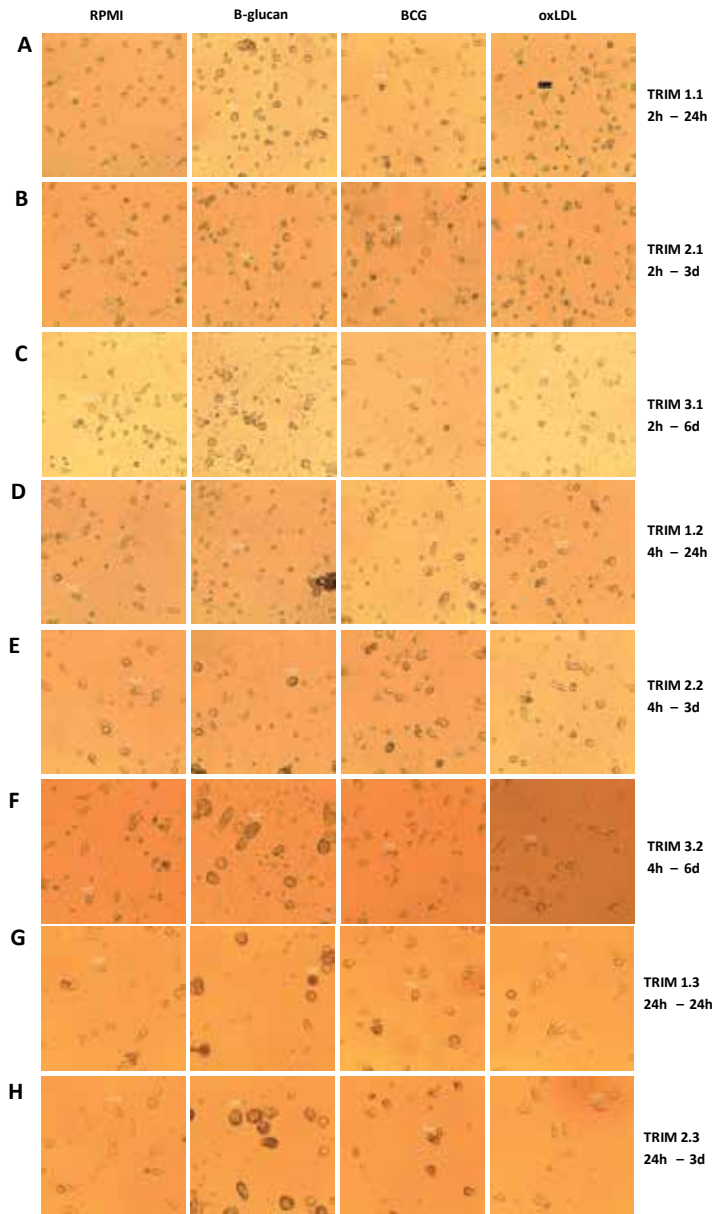




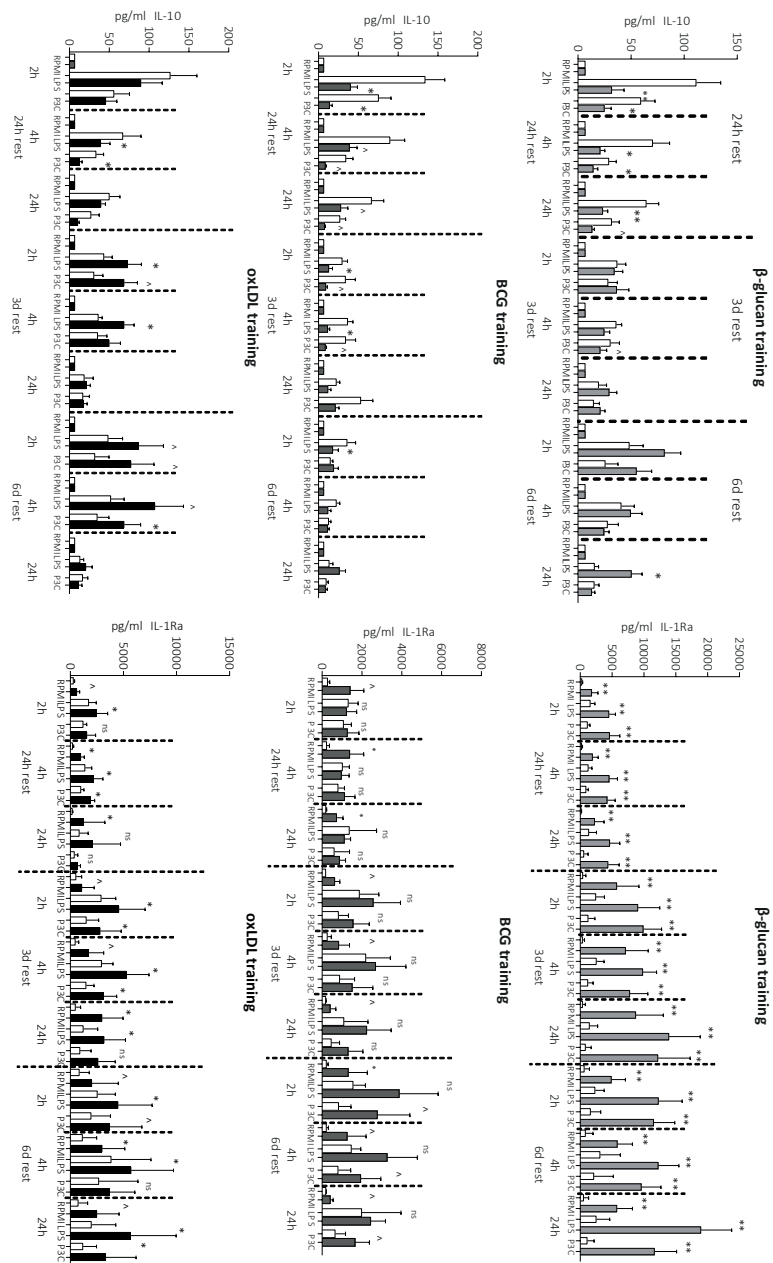
**Supplementary Figure 2.** Increased pro-inflammatory cytokine production is dependent on both training and resting time. (A) IL-6 production after re-stimulation in pg/ml; cells were trained for 2h, 4h or 24h with  $\beta$ glucan (upper panel), BCG (middle panel) or oxLDL (lower panel), and rested for 24h, 3d or 6d. (B) TNF $\alpha$  production after re-stimulation in pg/ml; cells were trained for 2h, 4h or 24h with  $\beta$ glucan (upper panel), BCG (middle panel) or oxLDL (lower panel), and rested for 24h, 3d or 6d (n=6, \* p<0.05, \*\* p<0.01, \*\*\* P<0.001)



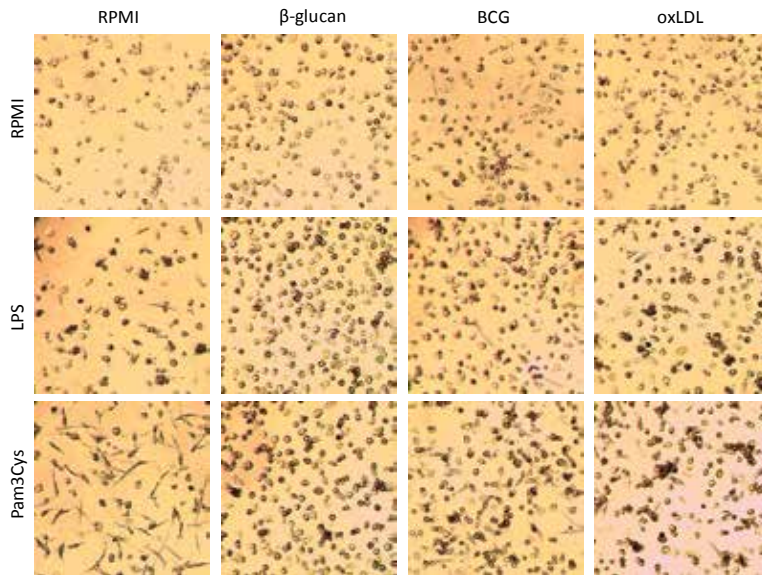
**.Supplementary Figure 3.** Training induces changes in cell morphology. (A) Cell morphology of cells from 2hr-T; 24hr-R trained with RPMI (negative control)  $\beta$ glucan, BCG or oxLDL. Pictures were taken before re-stimulation (20x). (B) Cell morphology of cells from 2hr-T; 3d-R (C) Cell morphology of cells from 2hr-T; 6d-R (D) Cell morphology of cells from 4hr-T; 24hr-R (E) Cell morphology of cells from 4hr-T; 3d-R (F) Cell morphology of cells from 4hr-T; 6d-R (G) Cell morphology of cells from 24hr-T; 24hr-R (H) Cell morphology of cells from 24hr-T; 3d-R



**Supplementary figure 4.** Anti-inflammatory cytokine production is increased in trained monocytes. (A) Production of the anti-inflammatory cytokine IL-10. (B). Production of the anti-inflammatory cytokine IL-1Ra increases upon training, not only after restimulation, but also on baseline. Cells were trained for 2h, 4h or 24h with  $\beta$ glucan (upper panel), BCG (middle panel) or oxLDL (lower panel), and rested for 24h, 3d or 6d (n=6, ^=p0,06 \* p<0.05, \*\* p<0.01, \*\*\* P<0.001).



**Supplementary Figure 5.** Morphological changes after 24hr training and 6 days resting persist after 24h of restimulation. Cell morphology of cells trained with either RPMI,  $\beta$ -glucan, BCG or oxLDL and restimulated with RPMI, LPS or Pam3Cys.





# CHAPTER 4

OX-LDL INDUCES LONG-TERM PRO-INFLAMMATORY  
CYTOKINE PRODUCTION AND FOAM CELL FORMATION VIA  
EPIGENETIC REPROGRAMMING OF MONOCYTES

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## ABSTRACT

### Objective

Although the role of monocytes in the pathogenesis of atherosclerosis is well-established, the persistent vascular inflammation remains largely unexplained. Recently, our group reported that stimulation of monocytes with various microbial products can induce a long-lasting pro-inflammatory phenotype via epigenetic reprogramming, a process termed ‘trained immunity’. We now hypothesize that oxidized LDL (oxLDL) also induces a long-lasting pro-inflammatory phenotype in monocytes, which accelerates atherosclerosis by pro-inflammatory cytokine production and foam cell formation.

### Approach and Results

Isolated human monocytes were exposed for 24 hours to medium or oxLDL. After washing and resting for 6 days, the cells were exposed to Toll-like Receptor 2 and 4 agonists. Pre-exposure to oxLDL increased mRNA expression and protein formation upon TLR2/4 stimulation of several pro-atherogenic proteins, including interleukin (IL)-6, IL-18, IL-8, tumor necrosis factor- $\alpha$ , monocyte chemoattractant protein-1 and matrix metalloproteinase 2, and 9. In addition, foam cell formation was enhanced after oxLDL exposure, which was associated with an upregulation of scavenger receptors CD36 and scavenger receptor-A and downregulation of ATP binding cassette transporters ABCA1 and ABCG1. Chromatin immunoprecipitation performed 6 days after oxLDL stimulation demonstrated increased trimethylation of lysine 4 at histone 3 in promoter regions of *tnf $\alpha$* , *il-6*, *il-18*, *mcp-1*, *mmp2*, *mmp9*, *cd36* and *sr-a*. Finally, pretreatment of the monocytes with the histone methyltransferase inhibitor methylthioadenosine completely prevented the oxLDL-induced long-lasting pro-inflammatory phenotype.

### Conclusions

Brief exposure of monocytes to a low concentration of oxLDL induces a long-lasting pro-atherogenic macrophage phenotype via epigenetic histone modifications, characterized by increased pro-inflammatory cytokine production and foam cell formation.

## INTRODUCTION

Atherosclerosis is a chronic inflammatory disorder of the arterial vessel wall, which is initiated by an accumulation of lipoproteins in the intimal layer of the vascular wall. Subsequent activation of the overlying endothelium leads to the recruitment of circulating monocytes<sup>1</sup>. Monocyte entry into the vessel wall is crucial for the onset of the disease, since mice lacking monocyte chemokines or their receptors have less atherosclerosis<sup>2-4</sup>. Within the intimal layer, monocytes differentiate into macrophages that subsequently engulf oxidized low density lipoprotein (oxLDL) particles via scavenger receptors, mainly CD36 and Scavenger Receptor-A (SR-A)<sup>5</sup>, leading to the formation of foam cells<sup>6</sup>. In addition, lesional macrophages can be activated by various stimuli, leading to the release of pro-inflammatory cytokines and matrix degrading proteins, thereby promoting disease progression and plaque destabilization<sup>7,8</sup>. This activation of macrophages occurs via membrane-bound and intracellular pattern recognition receptors (PRRs), including Toll-like receptor (TLR) 2 and -4. Interestingly, the rate of progression of atherosclerosis is critically dependent on the characteristics of the circulating monocyte pool. It has been established in mouse models of atherosclerosis that hypercholesterolemia and a myocardial infarction can accelerate atherogenesis by changing the number and characteristics of circulating monocytes<sup>9,10</sup>. Although the role of monocytes in atherosclerosis has now been well-established, it still remains elusive why the strong inflammatory response in the arterial wall persists in time.

Recently, our group reported that monocytes can build up an immunological memory after microbial stimulation via epigenetic reprogramming of histone modifications, and this mechanism has been termed 'trained immunity'<sup>11,12</sup>. In humans, monocytes isolated from healthy volunteers after *Bacillus Calmette–Guérin* (BCG) vaccination present an increased production of pro-inflammatory cytokines *ex-vivo* after re-exposure to various unrelated pathogens<sup>13</sup>. This enhanced pro-inflammatory state of the monocytes could be detected up to one year after first exposure<sup>14</sup>. In addition to BCG, 'trained immunity' can also be induced by *Candida albicans* and its cell wall component  $\beta$ -glucan<sup>15</sup>. It has been shown that trained immunity is at least partly mediated by an increased trimethylation of histone 3 at lysine 4 (H3K4me3), which is associated with an increased expression of target genes upon re-stimulation of the cells, including various pro-inflammatory cytokines. The kinetics of the epigenetic process leading to this sustained functional reprogramming of monocytes and macrophages has recently been described, and the epigenetic units responsible for this process have been termed "latent enhancers"<sup>16</sup>.



A genome wide analysis of the epigenetic changes induced by the exposure of monocytes to  $\beta$ -glucan revealed that the top-500 genes with a clear increase of H3K4Me3 include many genes that are involved in the process of atherosclerosis. Therefore, we recently hypothesized that trained innate immunity contributes to atherosclerosis<sup>12</sup>. We now investigate whether sustained epigenetic remodeling of monocytes can also be induced by non-microbial stimuli that are relevant in atherosclerosis, such as oxLDL. In addition, we characterize in detail the phenotype of the long-term activated monocyte-derived macrophage with regard to production of cytokines and chemokines that orchestrate the process of atherosclerosis and with regard to foam cell formation.

## MATERIALS AND METHODS

### Cells and reagents

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy volunteers (Sanquin Bloodbank Nijmegen, the Netherlands) by density-gradient centrifugation using Ficoll-Paque (GE Healthcare, Zeist, The Netherlands).  $\beta$ -glucan (b-1,3-(D)-glucan) was kindly provided by Professor David Williams (College of Medicine, Johnson City, USA). Stimuli and inhibitors used are lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO; from *E. coli* serotype 055:B5, further purified as described<sup>1</sup>) and LPS from *Bartonella*, further purified as described<sup>1</sup> as anti-TLR4, Pam3Cys (EMC microcollections, Tübingen, Germany; L2000), histone methyltransferase inhibitor methylthioadenosine (MTA, Sigma-Aldrich, D5011), histone demethylase inhibitor Pargyline (Sigma-Aldrich, P8013), Anti-TLR2 (Invivogen, maba-htlr2), IgG Isotype control (Invivogen, maba2-ctrl), SYK inhibitor (EMC microcollections, 574711), JNK inhibitor SP600125 (AG Scientific, S-2022), PI3K inhibitor 3MA (Sigma, M9281), ERK inhibitor U0126 (Promega, V112A), Cytochalasin B (Sigma, C6762), acetylated LDL (Bioconnect, BT906). Rabbit-anti-actin antibody (A5441) was purchased from Sigma; polyclonal rabbit anti-CD36 antibody (PA1-16813) was purchased from Thermo scientific, Pierce Antibodies; monoclonal mouse-anti-human MRS1/SR-A (MAB27081) was purchased from R&D systems (Minneapolis, MN).

### Training of adherent monocytes

Adherent monocytes were trained as described previously (depicted in figure 1)<sup>2</sup>. Briefly, monocytes were isolated from PBMCs by adhering  $5 \cdot 10^6$  PBMCs/ml to polystyrene for 1h at 37°C, 5% CO<sub>2</sub> in 96-, 24- or 6-well culture plates (Corning, New York, USA). Non-adherent cells were removed by washing with warm PBS. Monocytes were cultured in RPMI 1640 Dutch-modified culture medium (Life Technologies/Invitrogen, Breda, The Netherlands) supplemented with 10 mM glutamine (Invitrogen), 10  $\mu$ g/mL gentamicin (Centraform), 10 mM pyruvate (Invitrogen) and 10% pooled human serum.

The monocytes were incubated with  $\beta$ -glucan (1 or 5  $\mu$ g/ml), LDL (1-100  $\mu$ g/ml), oxLDL (1-100  $\mu$ g/ml) or acLDL (1-100  $\mu$ g/ml) for 24h, after which the cells were washed and re-incubated in culture medium. In inhibition experiments, cells were pre-incubated with the inhibitors 1h prior to adding the stimuli. Inhibitors used are either RPMI or RPMI+DMSO as a negative control, MTA (1 mM), Pargyline (3  $\mu$ M), aTLR2 (10  $\mu$ g/ml), IgG control (10  $\mu$ g/ml), aTLR4 (100 ng/ml), SYKi (50 nM), SP600125 (20  $\mu$ M), 3MA (20 mM), U0126 (10  $\mu$ M) and cytochalasin B (1  $\mu$ g/ml). After 7 days of incubation in supplemented RPMI medium, cells were exposed to either medium alone, 10 ng/mL LPS or 10  $\mu$ g/mL Pam3Cys for 24h at 37°C, 5% CO<sub>2</sub>. After 24h of incubation, supernatants were collected and stored at -20°C. Cell

death was assessed by measuring lactate dehydrogenase (LDH) in the supernatants using a Cytotox96® nonradioactive cytotoxicity assay (Promega Corporation, Madison, WI, USA). Production of pro-inflammatory cytokines was measured in supernatants by ELISA according to the manufacturers' instructions (IL-6, IL-8 (Sanquin, Amsterdam, The Netherlands); TNF $\alpha$ , IL-1 $\beta$  (R&D Systems, Minneapolis, USA)). The MCP-1 ELISA was performed according to the manufacturers' instructions using antibodies anti-Human CCL2 Purified (14-7099), anti-CCL2 Biotin (13-7096) and Human CCL2 Recombinant Protein (14-8398), all obtained from eBioscience (Halle-Zoersel, Belgium). Gene expression of these cytokines was measured as indicated below by qPCR.

### Quantitative Real-Time PCR

For qRT-PCR, monocytes were trained as described above and subjected to re-stimulation on day 7 with LPS (10 ng/ml) for 4 hours. At day 0 after adherence, at day 1 after 24h training and after re-stimulation at day 6, cells were stored in TRIzol reagent (Invitrogen). Total RNA purification was performed according to the manufacturer's instructions. RNA concentrations were measured using NanoDrop software, and isolated RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-rad, Hercules, CA). qPCR was performed using the SYBR Green method (Applied Biosciences, Carlsbad, CA). Used primers are listed in table 1 (Biolegio, Malden, The Netherlands). Samples were analyzed following a quantitation method with efficiency correction, and HPRT was used as a housekeeping gene. Relative mRNA expression levels of non-primed and non-stimulated samples were used as reference.

Gene	Forward primer	Reverse primer
HPRT	CCTGGCGTCGTGATTAGTGAT	AGACGTTCACTCCTGTCCATAA
IL-6	AACCTGAACCTTCCAAAGATGG	TCTGGCTTGTTCTCACTACT
TNF $\alpha$	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
MCP-1	CCAGTCACCTGCTGTTATAAC	TGGAATCCTGAACCCACTTCT
IL-18	TGTCGCAGGAATAAAGATGGCT	CCTTGGTCAATGAAGAGAACTTGGT
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCTCTGCACCCAGTTTTCT
MMP2	TACAGGATCATTGGCTACACACC	GGTCACATCGCTCCAGACT
MMP8	ACCAAAGAGATCACGGTGACA	TCCATGTTTCTTCGGCATCAAAA
MMP9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
CD36	CTTTGGCTTAATGAGACTGGGAC	GCAACAAACATCACCACACCA
SR-A/ MSR1	CCAGGTCCAATAGGTCTCC	CTGGCCTTCCGGCATATCC
ABCA1	GGTGATGTTTCTGACCAATGTGA	TGTCCTCATACCAGTTGAGAGAC
ABCG1	CGGAGCCCAAGTCGGTGTG	TTTCAGATGTCCATTACAGCAGGTC

TLR4	GGCATGCCTGTGCTGAGTT	CTGCTACAACAGATACTACAAGCACACT
TLR2	GAATCCTCCAATCAGGCTTCTCT	GCCCTGAGGGAATGGAGTTTA
HO1	AAGACTGCGTTCTGCTCAAC	AAAGCCCTACAGCAACTGTCTG
ARG1	GATTCCCGATGTGCCAGGATT	GCCAATTCTAGTCTGTCCACTT
CD163	TGAAGACTCTGGATCTGCTGA	GAAGTGGTGACAAAACAGGCA
iNOS	TTCAGTATCACAACTCAGCAAG	TGGACCTGCAAGTTAAATCCC
HLA-DRB1	CGGGGTTGGTGAGAGCTTC	AACCACCTGACTTCAATGCTG
DC-SIGN	TCAAGCAGTATTGGAACAGAGGA	CAGGAGGCTGCGGACTTTTT
CD83	AAGGGGCAAAATGGTTCTTTCG	GCACCTGTATGTCCCCGAG

**Table 1. Primer sequences used for qRT-PCR analysis (5'-3')**

### Preparation of oxidized LDL

Oxidized LDL was prepared by incubation with 20  $\mu\text{mol CuSO}_4/\text{L}$  for 15h at 37°C in a shaking water bath as described previously<sup>3,4</sup>. To induce foam cell formation at day 6, trained monocytes were first incubated in serum-free RPMI medium for 4h and subsequently incubated with RPMI medium alone or RPMI containing oxidized LDL at 50  $\mu\text{g}/\text{mL}$  for 24h. Incubation of primed monocytes with this concentration of oxLDL alone caused no secretion of IL-6, TNF $\alpha$  or MCP-1, suggesting absence of endotoxin in the prepared LDL fractions. Accumulation of oxidized LDL was visualized by Oil-Red-O staining as described<sup>5</sup> and the uptake was measured by ELISA, measuring intracellular apoB as described before<sup>6</sup>.

### Western blotting experiments

Scavenger receptor expression on trained monocytes was measured in cell lysates using Western blots. Monocyte training was performed as described above. On day 6, cells were lysed using lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM  $\beta$ -glycerophosphate, 50 mM sodium fluoride, 200  $\mu\text{M}$  sodium orthovanadate, complete mini EDTA-free protease inhibitor cocktail (Roche), and PhosphoSTOP Phosphatase Inhibitor Cocktail (Roche)). Total protein concentration of lysates was measured using a Pierce® BCA protein assay (Thermo Scientific, Rockford, IL) and 10% Tris-HCl gels were loaded with equal amounts of protein per lane (approx. 15  $\mu\text{g}$ ). Proteins were blotted onto a nitrocellulose membrane and the membrane was subsequently blocked with 5% milk (w/v) in Tris-buffered saline supplemented with Tween 20 (TBS-T). Primary antibodies (Rabbit-anti-CD36 1:1000, mouse-anti-SR-A 1:1000 and Rabbit-anti-Actin 1:1000) were incubated in 5% milk in TBS-T overnight at 4°C. After three times washing with TBS-T, HRP-conjugated secondary antibodies were incubated for 1h at room temperature (RT, 1:5000 Swine-anti-rabbit-HRP or 1:5000 Rabbit-anti-mouse-HRP) in 5% ELK in TBS-T.

After three times washing, chemiluminescence was measured using ECL Plus Western Blot Detection Reagents (Amersham Biosciences, UK) and the Chemidoc XRS imager with Image Lab software (Bio-Rad, CA, USA).

### Chromatin Immunoprecipitation

For the assessment of H3K4me3, ChIP was performed as described previously<sup>7</sup>. In short, chromatin of cells on day 6 was fixated in methanol free 1% formaldehyde (28908, Thermo Scientific), followed by sonication and immunoprecipitation using antibodies against H3K4me3 (Diagenode, Seraing, Belgium). ChIPed DNA was processed further for qPCR analysis. Primers used in the reaction are listed in table 2. Samples were analyzed following a comparative Ct method, myoglobin was used as a negative control and H2B as a positive control according to the manufacturer's instructions.

Promotor of gene	Forward primer	Reverse primer
Myoglobin	AGCATGGTGCCACTGTGCT	GGCTTAATCTCTGCCTCATGAT
H2B	TGTACTIONGGTGACGGCCTTA	CATTACAACAAGCGCTCGAC
TNF $\alpha$	CAGGCAGGTTCTCTCTCTCT	GCTTTCAGTGCTCATGGTGT
IL-6	TCGTGCATGACTTCAGCTTT	GCGCTAAGAAGCAGAACCAC
IL-18	ATGCACTGGGAGACAATTCC	CTCCCTCCACCTTCTTCTC
MCP-1/CCL2	CCGAGAGGCTGAGACTAACC	CTATGAGCAGCAGGCACAGA
CD36	TCACGCATTATGTACTGAGGA	TGCTCCAACATTTGGACAAT
SR-A/MSR1	GCATCTGGTACATGAAGAAAGG	TGTGCATTGATGAGAGTGCT
MMP2	CTGCATCCAGACTTCCTCAG	GTCCTGGCAATCCCTTTGTA
MMP8	GAGCAGAAATGGAAGCGTCT	AAAGAAAGCCAGGAGGGGTA
MMP9	CAGTCCACCCTTGTGCTCTT	CTCTGCCAGCTGCCTGTC

*Table 2. Primers used for ChIP qPCR analysis (5'-3')*

### Statistical analysis

Each experiment was performed at least 6 times. Data are presented as means  $\pm$  SD. For Oil-Red staining and Western blotting, representative pictures are shown. Group comparisons were performed using the Wilcoxon signed-rank test. P-values < 0.05 (\*) are considered significant; (\*\*) p < 0.01; (\*\*\*) p < 0.001.

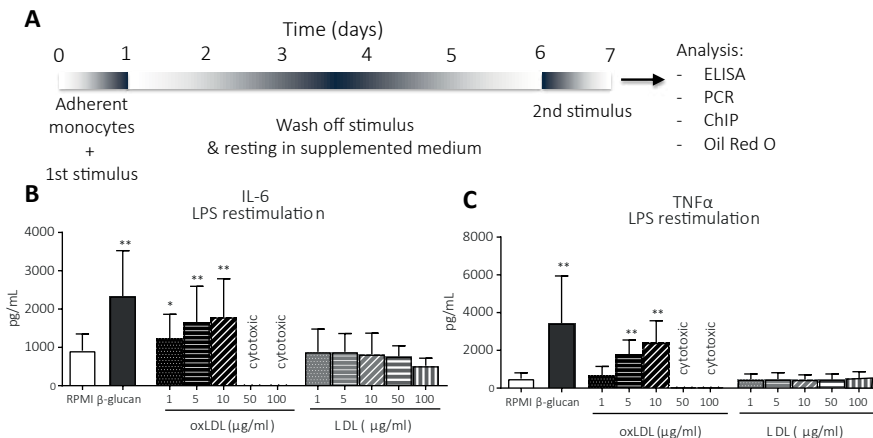
## RESULTS

### $\beta$ -glucan training induces enrichment of H3K4me3 on genes relevant for atherosclerosis

From our previous work on monocyte training with  $\beta$ -glucan, we analyzed the top 500 H3K4Me3 enriched genes for their involvement in the process of atherosclerosis<sup>15</sup>. These genes not only involved various cytokines and chemokines that are relevant in atherogenesis, but also scavenger receptors and other proteins involved in foam cell formation and proteins involved in atherosclerotic plaque destabilization (Supplementary Table I).

### OxLDL but not LDL induces training of monocytes in vitro

Using the established in vitro model of human primary monocyte training, we explored the effect of LDL and oxLDL on training (Figure 1A). Incubation of monocytes with LDL and oxLDL did not induce cytokine production (IL-6, TNF $\alpha$  or IL-1 $\beta$ ) in the first 24h stimulation-period (Suppl. Figure I B-D). Moreover, incubation with oxLDL in a concentration up to 10  $\mu$ g/ml and incubation with LDL (up to 100  $\mu$ g/ml) was not cytotoxic, as demonstrated by a Lactate Dehydrogenase (LDH) cytotoxicity assay (Supplementary figure I A). However, incubation of cells with oxLDL on day 1 induced a significant dose-dependent increase in IL-6 and TNF $\alpha$  production by monocytes that were re-stimulated with TLR ligands on day 6. A significantly increased cytokine production was observed for stimulation of both TLR4 (Lipopolysaccharide (LPS), figure 1B and C) and TLR2 (Pam3Cys, Supplementary figure II A-B). Interestingly, pre-



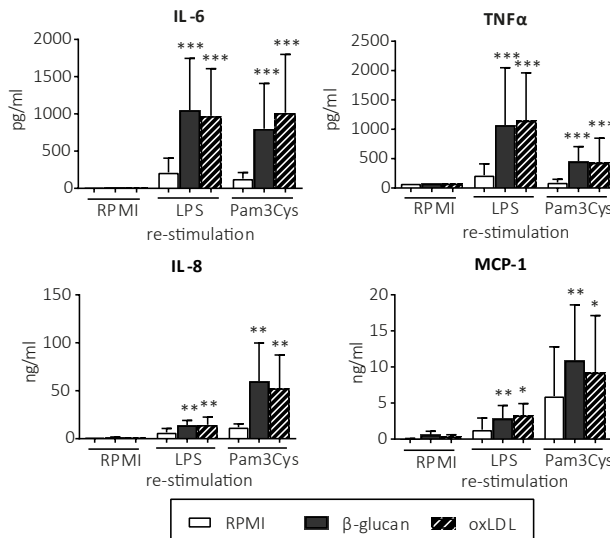
**Figure 1:** Priming with low concentrations of oxLDL but not native LDL augments IL-6 and TNF $\alpha$  production upon re-stimulation with LPS. (A) Schematic representation of the in vitro experimental setup. (B) IL-6 and (C) TNF $\alpha$  production was measured in the supernatants of adherent monocytes from healthy volunteers, exposed for 24h to either medium alone,  $\beta$ -glucan as a positive control, or different concentrations of oxidized LDL or native LDL. After washing and six days of resting period, monocytes were re-stimulated with LPS (n=8, \* P<0.05, \*\* p<0.01 compared to the RPMI control).

incubation with native LDL in a concentration up to 100 µg/ml did not have any effect on the capacity of monocytes to produce IL-6 and TNFα upon re-stimulation, suggesting that the modification of LDL is required to induce training. In an additional set of experiments, oxLDL was kept in the medium for the entire 6-day resting period. Similar potentiating effects on the inflammatory profile of monocytes were obtained (Supplementary figure III).

To assess whether training could also be induced by alternative modification of LDL, we repeated the experiments with acetylated LDL (acLDL), which also binds SR-A but not CD36. Indeed, re-stimulation of the cells on day 6 with either TLR4 or TLR2 agonists induced an increase in IL-6 and TNFα production, similar to oxLDL, albeit higher concentrations of acLDL were required (Supplementary figure II C).

### Characteristics of oxLDL-induced trained monocyte-derived macrophages

In our experimental design, after 24h exposure to oxLDL, the monocytes were kept in RPMI culture medium supplemented with 10% serum for 6 additional days until re-stimulation on day 6. During this period, the monocytes differentiate into monocyte-derived macrophages. We have previously reported that training with β-glucan did not induce skewing of the macrophages into type M1 or M2 macrophages<sup>15</sup>. In addition, monocytes can also differentiate into dendritic cells (DCs), such as TNF and nitric oxide synthase (iNOS) producing DCs (Tip-DC)<sup>17</sup>. It should be realized that this categorization into distinct macrophage phenotypes likely represents a simplified model since it recently a spectrum of macrophage activation states was revealed upon stimulation with various stimuli, extending the current M1 versus

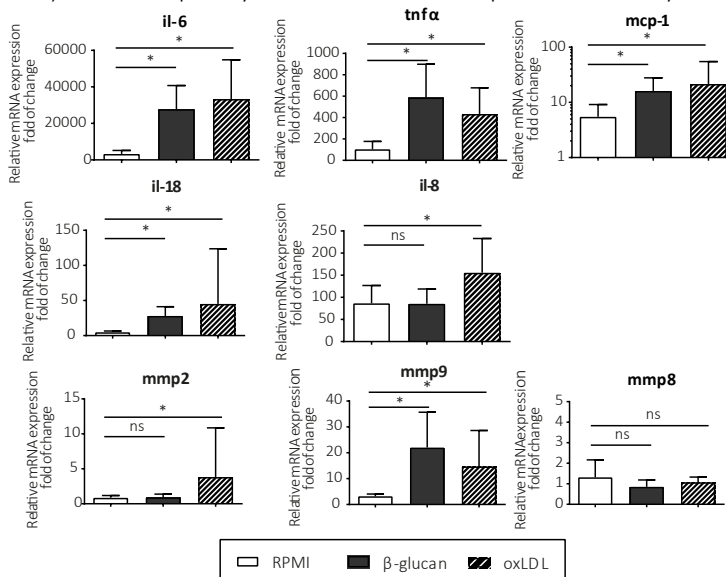


**Figure 2:** Primed monocytes have a more atherogenic cytokine and chemokine profile upon re-stimulation than non-primed monocytes. β-glucan and oxLDL primed monocytes produce more IL-6, TNFα, IL-8 and MCP-1 upon re-stimulation than untrained monocytes. Cytokine levels are measured in supernatants of cultured adherent monocytes from healthy volunteers, trained by either β-glucan, oxLDL or medium alone for 24h, and re-stimulated after 6 days with TLR2 or TLR4 agonists. (n≥10 per group, \* p<0.05, \*\* p<0.01, \*\*\* p<0.005 compared to the RPMI control)

M2-polarization model<sup>18</sup>. To better characterize the phenotype of the monocytes-derived macrophages on day 7 after stimulation with oxLDL, we measured the expression of several markers of classically activated and alternatively activated macrophages, and of inflammatory Tip-DC's on day 7 (Suppl. Fig. IV). We observed no significant differences in expression of TLR4, TLR2 and iNOS (M1 markers), Arginase1 and CD163 (M2 markers), and HLA-DR, DC-SIGN and CD83 expression (DC markers).

### Training of monocytes induces an atherogenic cytokine and chemokine response upon secondary stimulation

A broad spectrum of cytokines and chemokines have been implicated to play a role in atherogenesis, including IL-6, IL-18, TNF $\alpha$ , Monocyte Chemoattractant Protein 1 (MCP-1) and IL-8<sup>19</sup>. To study the effect of brief exposure to oxLDL on the production of these cytokines and chemokines, monocytes were trained for 24h by either medium alone as a negative control,  $\beta$ -glucan as a positive control, or oxLDL. After washing on day 1 and the additional six days resting period, cells were re-stimulated by LPS and Pam3Cys for 4h or 24h to study the induction of mRNA production and cytokine or chemokine production, respectively. Both  $\beta$ -glucan and oxLDL training of monocytes augmented the production of IL-6, TNF $\alpha$ , IL-8 and MCP-1 (Figure 2). We subsequently assessed whether the potentiation of cytokine production



**Figure 3:** mRNA expression of pro-atherogenic cytokines is enhanced in trained monocytes. Adherent monocytes of healthy volunteers were exposed to either  $\beta$ -glucan, oxLDL or medium alone for 24h, and re-stimulated after 6 days with LPS for 4h. Relative mRNA expression values are shown, compared to the untrained control. Expression of il-6, tnfa, mcp-1, il-8, il-18, mmp2 and mmp9 but not mmp8 in trained monocytes is significantly higher on upon re-stimulation then in untrained monocytes ( $n=6$  per group, \*  $p<0.05$ )

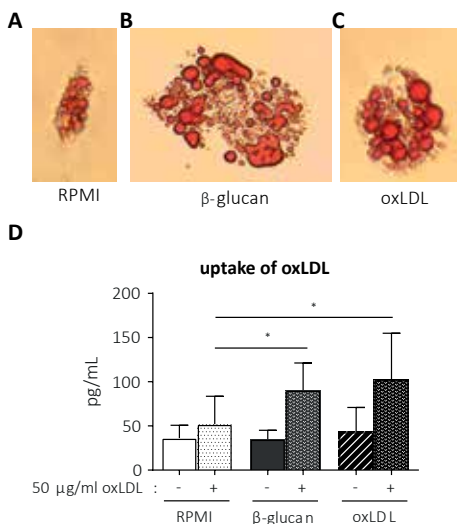


is exerted at the gene transcription level. Upon re-stimulation of trained monocytes, il-6, tnfa, il-8, mcp-1 and also il-18 mRNA expression showed a significant up-regulation compared to the untrained control (Figure 3A-E). Interestingly, whereas the basal levels of il-6, tnfa and il-8 showed no difference upon training, the baseline expression of il-18 was significantly upregulated (Suppl. Fig V) and the baseline expression of mcp-1 shows a similar effect, albeit not significantly, suggesting enhanced constitutive expression of these genes.

In addition to enhanced cytokine production, we also observed an enhanced baseline mRNA expression of Matrix Metalloproteinase 9 (MMP9), and increased expression of MMP2 and -9 upon LPS re-stimulation, two of the most important matrix degrading proteins, responsible for plaque destabilization (Fig 3E, G). In contrast, MMP8 was not upregulated at baseline or upon re-stimulation (Fig 3F and Suppl. Fig. V). The upregulation of mmp2 and mmp9, and not mmp8 mRNA is in accordance with our previous findings on H3K4Me3 enrichment in the promoter regions of MMP2 and 9, but not MMP8 after training with  $\beta$ -glucan (Suppl. Table I).

### Foam cell formation is enhanced in trained monocytes-derived macrophages

In the vessel wall, the uptake of oxLDL by macrophages leads to the generation of foam cells, thereby contributing to the formation of atherosclerotic plaques<sup>6</sup>. Therefore, we explored foam cell formation of trained monocyte-derived macrophages in our in-vitro model. For this purpose, we trained adherent monocytes and subsequently induced foam cell formation on day 6 by incubation with a high dose of oxLDL. Oil Red O staining revealed an increased intracellular accumulation of lipid droplets in cells stimulated with oxLDL and  $\beta$ -glucan compared to control cells (Figure 4A-C). The first 24h priming of monocytes with a low dose of oxLDL did not induce foam cell formation (data not shown). To quantify the uptake of oxLDL by foam cells, intracellular apolipoprotein B (ApoB) levels were measured and compared to the

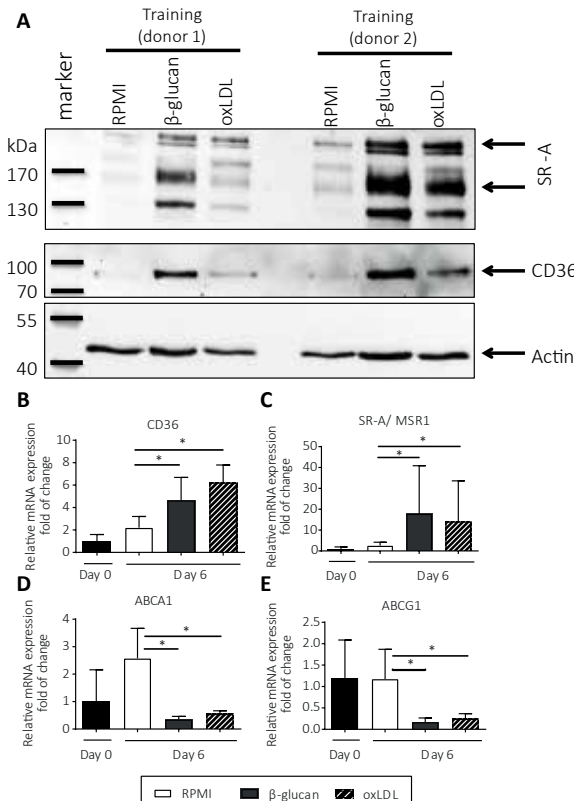


**Figure 4:** Foam cell formation is significantly enhanced in trained monocytes in vitro. Adherent monocytes of healthy volunteers were exposed to  $\beta$ -glucan, oxLDL or medium alone for 24h and rested for 6 days in supplemented medium. 4h before foam cell induction, the cells were starved from serum and were subsequently incubated with a high amount of oxLDL (50  $\mu$ g/mL) for 24h. Intracellular apoB levels were measured by ELISA (D) and foam cell morphology of trained monocytes was compared to untrained monocytes by staining with Oil Red O (A-C, single cell, 40x; A. RPMI, B.  $\beta$ -glucan, C. oxLDL) (representative pictures, n=6, \* p<0.05)

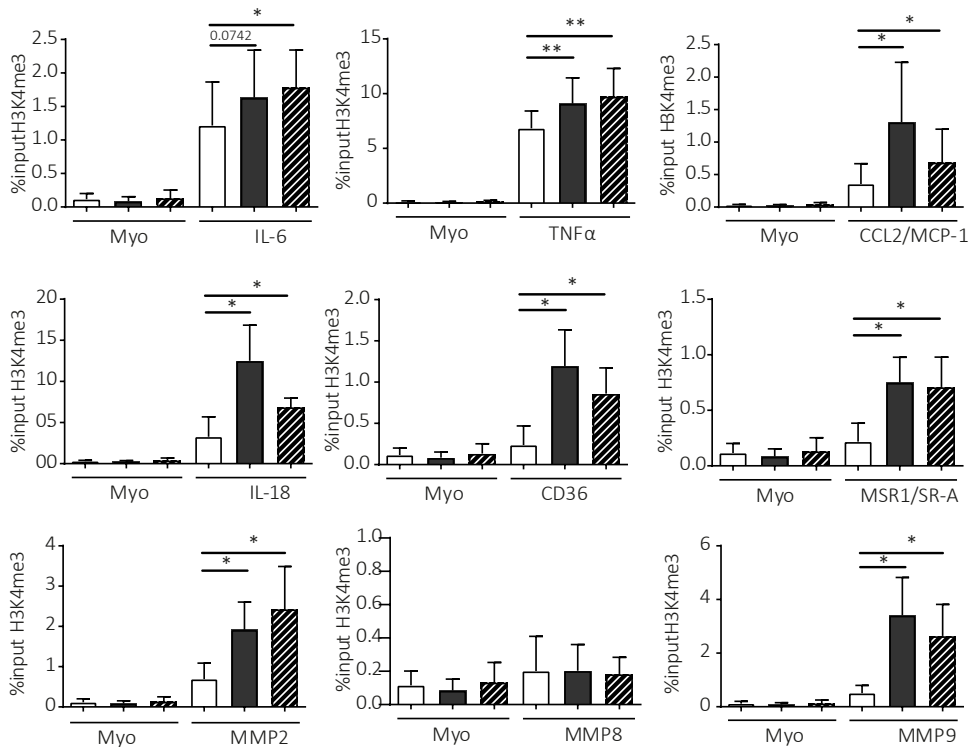
medium control. Both  $\beta$ -glucan and oxLDL exposure significantly increased the intracellular ApoB concentration on day 6 (Figure 4D).

### Trained monocytes have an increased expression of scavenger receptors and reduced expression of cholesterol efflux transporters

OxLDL is taken up by macrophages via scavenger receptors, of which CD36 and Scavenger Receptor A (SR-A/MSR-1) are considered the most important<sup>5</sup>. Interestingly, the genes of these receptors showed enriched H3K4me3 in our previous study of training with  $\beta$ -glucan (Suppl. Table 1). To study the expression of these scavenger receptors, we trained adherent monocytes with oxLDL and on day 6 the cells were processed for analysis by Western blot. We found that both CD36 and SR-A were strongly up-regulated in trained monocytes in comparison to untrained controls (Fig. 5A). RNA expression analysis also showed up-regulation of gene expression CD36 and SR-A (Fig 5B-C). Because foam cell formation represents the net effect of cholesterol influx and efflux, we also studied the expression of the cholesterol efflux transporters ATP Binding Cassette Transporters ABCA1 and ABCG1. For both transporters, there was a significant down-regulation of mRNA expression (Fig 5D-E).



**Figure 5:** Expression of scavenger receptors CD36 and SR-A is increased on trained monocytes. Adherent monocytes of healthy volunteers were exposed to either medium,  $\beta$ -glucan or oxLDL for 24h. After six days, cells were lysed and equal protein concentrations were put on SDS-PAGE. Arrows indicate the proteins CD36 (~86 kDa), SR-A (~220-250 kDa, depending on dimerization/trimerization) and actin as control. Two different healthy volunteers are shown, pictures are representative of at least 6 donors. Protein expression for both receptors was enhanced for  $\beta$ -glucan training and oxLDL training, compared to the untrained control (A). Subsequently, mRNA expression of CD36 (B) and SR-A (C) was measured in cells on day 0 and day 6, and show increased mRNA expression of the scavenger receptors. Cholesterol efflux transporters ABCA1 (D) and ABCG1 (E) show downregulated mRNA expression upon training. (n=6, \* p<0.05)



**Figure 6:** Training induces enriched H3K4me3 on the promoters of pro-atherogenic genes. Chromatin Immunoprecipitation of histone 3 lysine 4 trimethylation (H3K4me3) was performed on chromatin from adherent monocytes from healthy volunteers, which were trained as described previously. Quantification of H3K4me3 was performed by qPCR analysis of the promoters of TNFα, IL-6, IL-18, MCP-1, MMP2, MMP8, MMP9, CD36 and SR-A. Results are shown as %input and myoglobin was used as a negative control. Significant upregulation of H3K4me3 on the promoters of TNFα, IL-6, IL-18, MCP-1, MMP2, MMP9, CD36 and SR-A but not MMP8 was shown for β-glucan training as well as oxLDL training (n=6, \* p<0.05, \*\* P<0.01, compared to the untrained RPMI control)

## Enriched H3K4me3 on the promoters of pro-atherogenic genes by oxLDL exposure

Our previous reports showed that training of monocytes by β-glucan increased H3K4me3 at the level of the promoters of pro-inflammatory cytokine genes. Here, we investigated whether oxLDL training also induced H3K4 trimethylation on genes encoding for pro-inflammatory cytokines, chemokines, MMPs or scavenger receptors. Therefore, we trained adherent monocytes as described above and performed chromatin immunoprecipitation (ChIP) on H3K4me3 on day 6, to assess sustained epigenetic changes. The %input H3K4me3 on the promoters of TNFα, IL-6, MCP-1, IL-18, MMP2, MMP8, MMP9, CD36 and SR-A were measured and compared to the untrained control. We confirmed enrichment of H3K4me3 on the promoters of TNFα, IL-6, MCP-1, IL-18, CD36, SR-A, MMP2 and MMP9 but not MMP8,

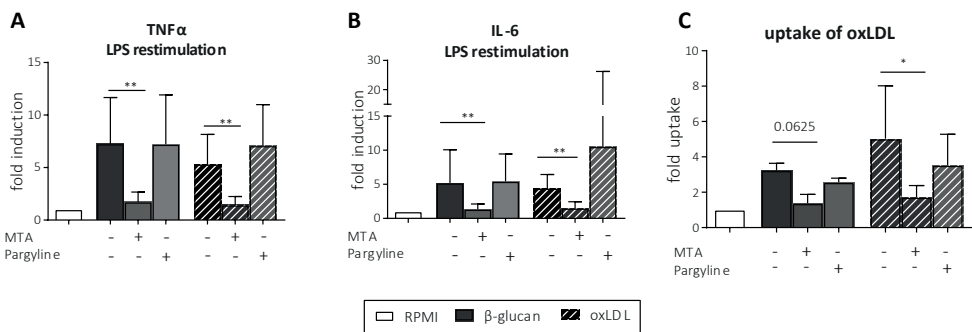
which correlates with the mRNA expression data (Fig. 6).

### OxLDL training of monocytes is dependent on methyltransferases

Finally, to show that training by oxLDL is indeed dependent on histone methylation, we used the nonselective pharmacological blocker of histone methyltransferases methylthioadenosine (MTA). We pre-incubated adherent monocytes with either culture medium or 1 mM of MTA, 1h before adding either culture medium,  $\beta$ -glucan or oxLDL. After a 24h incubation period, washing and 6 day resting, the cells were re-stimulated on day 6 with LPS and Pam3Cys. As a control, cells were also pre-incubated with the nonselective demethylase inhibitor Pargyline (3  $\mu$ M). IL-6 and TNF $\alpha$  concentrations were measured in the supernatant and compared to the untrained control (Figure 7A-B and Suppl. Fig VI A-B). Indeed, pretreatment with MTA completely abolished the training induced by oxLDL, whereas pargyline did not affect the training. Similarly, the augmented foam cell formation on day 6 after training with oxLDL was completely abolished by pretreatment with MTA (Fig. 7C).

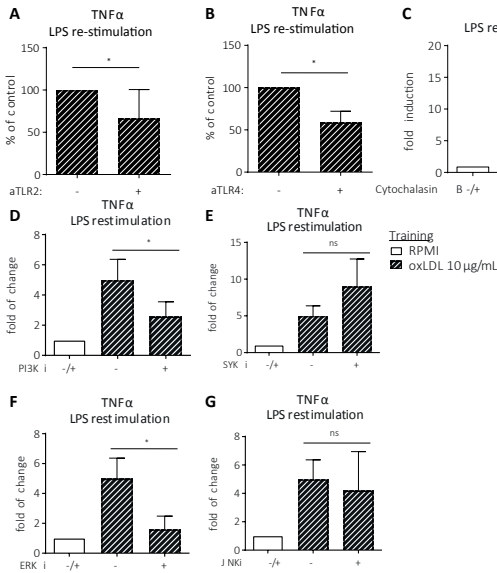
### Training of oxLDL is induced via a Toll-Like Receptor pathway

oxLDL can activate innate immune cells via TLR4, -2, -6 and CD36 heterodimerization<sup>20</sup>. To assess the mechanisms of oxLDL training, we pre-incubated the cells with TLR4 and TLR2 blockers before exposure to oxLDL on day 1. Pretreatment of the cells with both TLR4 and TLR2 blockers showed a partial reduction of the training effect (Figure 8A-B). Next, we examined which intracellular pathways are responsible for the training effect. Therefore, we pretreated the cells with pharmacological inhibitors of several intracellular pathway proteins, which are known to be involved in the TLR pathways, including ERK, PI3K, SYK and JNK. Pretreatment of



**Figure 7:** Methyltransferase inhibition prevents training by oxLDL. TNF $\alpha$  (A) and IL-6 (B) levels were measured in supernatants of adherent monocytes primed for 24h with either  $\beta$ -glucan as a positive control, oxLDL or medium alone in the absence or presence of the histone methyltransferase inhibitor MTA and the histone demethylase inhibitor Pargyline. At day 6, cells were re-stimulated with LPS. Next, foam cell formation was measured after pre-treatment of the cells with histone methyltransferase inhibitor MTA (C). Results are shown as fold induction, compared to the untrained control group. (n=8 per group, \*  $p<0.05$ , \*\*  $p<0.01$ )

the cells with inhibitors of ERK and PI3K significant reduced the cytokine production on day 6, whereas inhibition of SYK and JNK showed no effect. (Figure 8D-G). Finally, we excluded that CD36-mediated endocytosis of oxLDL is involved, by demonstrating that pretreatment of the cells with cytochalasin B, an inhibitor of actin polymerization, did not inhibit the training (Figure 8C).



**Figure 8: Pathway analysis of oxLDL training.** TNFα levels were measured in supernatants of adherent monocytes primed for 24h with either β-glucan as a positive control, oxLDL or medium alone in the absence or presence of TLR4 or TLR2 inhibitors (A-B), cytochalasin B (C) or several intracellular pathway inhibitors (D-G). At day 6, cells were re-stimulated with LPS. Results are shown as %change in training or fold of change and compared to the untrained control group. (n=5 per group, \* p<0.05)

## DISCUSSION

In this study, we demonstrate that macrophages derived from monocytes that have been exposed to modified forms of LDL, including oxLDL, show a long-term pro-inflammatory phenotype, due to epigenetic reprogramming of histones. These findings provide a novel mechanism that can contribute to the persistent vessel wall inflammation that is characteristic of atherosclerosis and can provide novel pharmacological targets to treat patients with atherosclerosis.

It is generally accepted that mononuclear phagocytes play a central role in the various stages of atherosclerosis<sup>8</sup>. The prevailing paradigm has long dictated that monocytes and macrophages do not have any immunological memory, in contrast to cells of the adaptive immune system. This assumption was recently challenged by the observation that 24 hours exposure of human monocytes to *C. albicans*,  $\beta$ -glucan or BCG induced long-term activated macrophages by epigenetic reprogramming of their transcriptional programs<sup>13-15</sup>. Analysis of markers for M1 and M2 macrophages suggested global pan-activation of these cells rather than simple skewing into M1 or M2 subtypes<sup>15</sup>. Since an analysis of the top 500 regions of enriched H3K4me3 after stimulation with these microbial products revealed many genes that are of importance in the initiation and progression of atherosclerosis (Suppl. Table I), we recently argued that this mechanism of trained innate immunity could explain the persistent vessel wall activation in atherosclerosis<sup>12</sup>.

OxLDL is crucial for the development of atherosclerosis by inducing cytokine and chemokine production in macrophages by stimulation of membrane-bound CD36 and TLR's 2, 4, and 6<sup>20,21</sup>. In addition, in isolated endothelial cells, oxLDL promotes IL-8 and MCP-1 secretion by increased acetylation of histone H3 and H4<sup>22</sup>. Based on these findings, we have now demonstrated that 24h exposure of isolated human monocytes to a low concentration of oxLDL induces the formation of macrophages with a long-lasting pro-inflammatory phenotype. These cells have several characteristics that can contribute to the formation of atherosclerotic plaques. By increased production of IL-6, TNF $\alpha$ , IL-8, IL-18 and MCP-1, these cells can promote and maintain inflammation in the vessel wall. Moreover, by up-regulation of scavenger receptors CD36 and SR-A and reduced expression of cholesterol export transporters ABCA1 and ABCG1, foam cell formation is augmented in these cells. Finally, increased production of MMP2 and MMP9 can contribute to destabilization of the atherosclerotic plaque. This long-lasting pro-atherogenic phenotype induced by oxLDL resulted from enrichment of H3K4Me3 in the promoter regions of the various cytokines, chemokines and transporters and was prevented by pharmacological inhibition of histone methyltransferases.

mRNA analysis of oxLDL-stimulated monocyte-derived macrophages revealed no significant changes in expression of M1, M2 or DC markers. These findings argue against simple skewing of these cells into pro-inflammatory classically activated macrophages or pro-inflammatory dendritic cells, as previously also described for stimulation of monocytes with  $\beta$ -glucan<sup>15</sup>. Pathway analyses with pharmacological inhibitors revealed that the effects of oxLDL are dependent on stimulation of TLR2 and 4. Experiments with the inhibitor of actin polymerization cytochalasin B demonstrated that CD36-mediated endocytosis of oxLDL is not required for the training of monocytes to occur. In addition, the observation that acLDL, which also binds SR-A but not CD36 suggests that CD36 is not needed to induce this biological effect<sup>23</sup>. Further characterization of the signaling pathways revealed that ox-LDL-induced training is critically dependent on activation of the MAP kinases ERK1/2 and PI3-K, but not SYK and JNK.

Our findings have three important implications. Firstly, our results increase our understanding of atherogenesis by revealing a novel mechanism that can contribute to the persistent inflammation of the vessel wall that is characteristic for atherosclerosis. Secondly, this mechanism might offer a novel explanation for the association between infections and atherosclerosis<sup>24</sup>. Interestingly, administration of BCG, which has been shown to induce training of monocytes<sup>13</sup>, to rabbits fed a cholesterol-enriched diet augments the formation of atherosclerosis<sup>25</sup>. Based on these findings it is tempting to speculate that exposure to micro-organism such as *C. pneumoniae* induces histone modifications in circulating monocytes, which subsequently home to the arterial endothelial lining and promote atherogenesis. In endothelial cells, exposure to *C. pneumoniae* induces inflammatory gene expression via acetylation of histone H4 and phosphorylation and acetylation of histone H3<sup>26</sup>. Finally, our results provide novel pharmacological targets to prevent or slow down the process of atherosclerosis: histone modifications are not static but amenable to (pharmacological) modulation by interfering with histone methyltransferases and histone demethylases. Particularly in the field of hematology and oncology, nonspecific methyltransferase inhibitors and histone deacetylase inhibitors are already used in clinical trials<sup>27</sup>. As a proof-of-concept Choi et al showed that pharmacological modulation of histone acetylation can indeed modulate the development of atherosclerosis<sup>28</sup>. Treatment of LDL-R<sup>-/-</sup> mice with the histone deacetylase inhibitor Trichostatin A increased atherosclerotic lesion size, and increased macrophage accumulation in aortic sinus.

A few limitations of our study need to be discussed. Firstly, to prevent H3K4Me3 in our experiments, we used MTA, which is a nonselective histone methyltransferase inhibitor. More specific inhibitors of H3K4Me3 are currently not available. To exploit our findings to develop novel pharmacological strategies in the treatment of atherosclerosis, the development of

these more specific inhibitors is critical. Secondly, we only studied H3K4Me3, which is only one of the epigenetic marks associated with an open transcriptionally active chromatin. Additional experiments are required to fully understand the epigenetic pathways involved in oxLDL-induced training of monocytes. Finally, our study only involves in-vitro stimulation of isolated human monocytes. It needs to be emphasized that we used LDL that was oxidized in vitro using copper sulphate, which probably does not represent fully the in vivo modified LDL in the vessel wall. In addition, acLDL does not occur in vivo and we cannot exclude additional oxidation of acLDL during the 24 hour incubation period. Therefore, to translate our findings to clinical practice, it is critical to perform future studies to investigate whether the epigenetic marks can be identified in patients with risk factors for atherosclerosis or patients with established atherosclerosis.

In conclusion, we provide the first in vitro evidence that oxLDL exposure of human monocytes induces epigenetic histone modifications that result in a long-lasting pro-inflammatory phenotype with increased production of pro-inflammatory cytokines and chemokines, augmented foam cell formation, and increased production of MMPs. Future studies both in animal models and in humans are necessary to provide definitive evidence that this mechanism affects the development of atherosclerosis and is amenable to pharmacological modulation.



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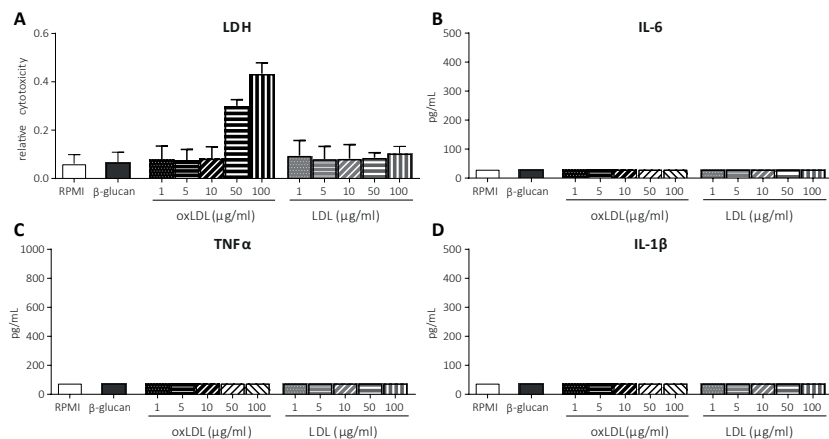
## SUPPLEMENTARY FIGURES

Type	Gene	Function
Cytokines and chemotactic proteins	IL-6 MCP-1/CCL2 TNF $\alpha$ TGF $\beta$ IL-18 Osteopontin/SPP1 AIF1	Inflammation, induction of SMC proliferation
Scavenger receptors and foam cell formation	CD36 MSR-1 SOAT1, ACAT2	Uptake of oxLDL
Matrix degradation, plaque instability	Cathepsin B Cathepsin H Cathepsin S Cathepsin L A2M MMP9 MMP2 ADAM9	Matrix degradation, plaque instability, reduction of cholesterol efflux in macrophages
Other inflammatory	CD63 LTA4H CD74 LGALS3 CD18	

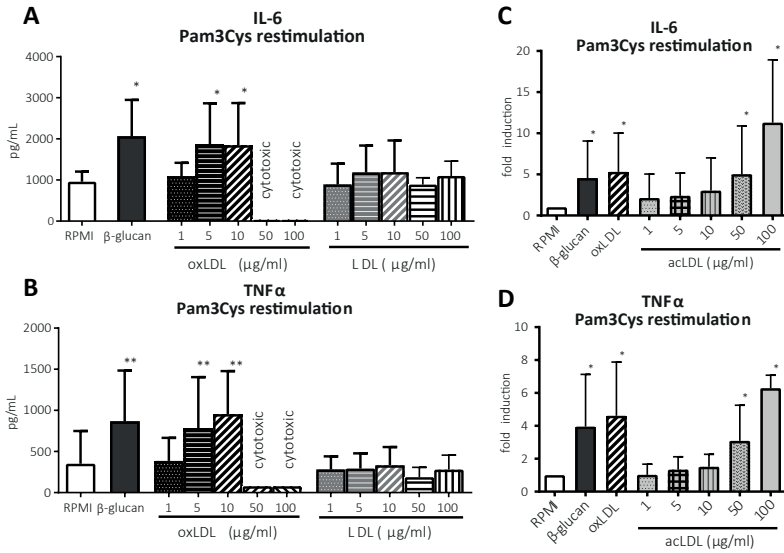
Other	LP-PLA2 Lipase A LPL FABP4, AQP9, PFN1, ACSL1 HSP90 GRN, LAMP2 PIIB CRABP2 S100A4 IFI30 ACP5 CFL1 ALDOa	Lipases induce the oxidation and enzymatic modification of LDL
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**Supplementary Table I.**  $\beta$ -glucan training induces H3K4me3 enrichment of various genes that are relevant for the process of atherosclerosis

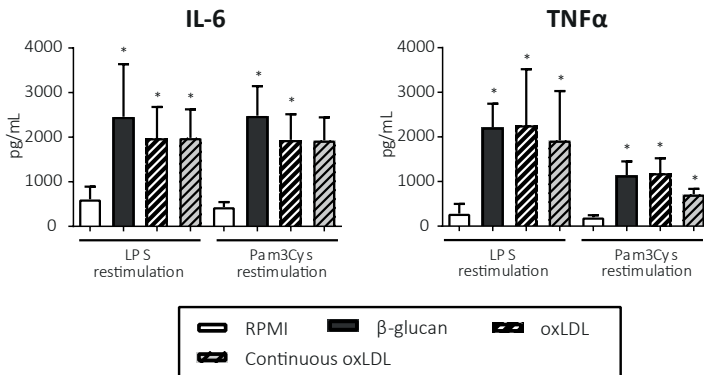
**Supplementary Figure I.** Cytotoxicity and stimulatory effects of LDL and oxLDL. The supernatant of the first 24h incubation period was studied for the release of LDH as a result of cytotoxic cell death (A). No significant increase of LDH release was observed for either  $\beta$ -glucan, LDL or oxLDL up to 10  $\mu\text{g/ml}$ . 50 and 100  $\mu\text{g/ml}$  oxLDL induced cytotoxic cell death ( $n=5$ ). Incubation with these stimuli did not induce any cytokine production itself (IL-6, TNF $\alpha$  and IL-1 $\beta$ ) at  $t=24\text{h}$ .



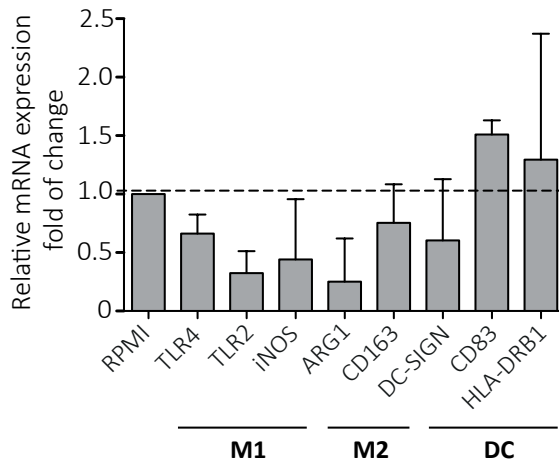
**Supplementary Figure II.** Priming with low concentrations of oxLDL and acetylated LDL but not native LDL can induce upregulated IL-6 and TNF $\alpha$  production upon re-stimulation with Pam3Cys. IL-6 and TNF $\alpha$  production was measured in the supernatants of adherent monocytes from healthy volunteers, exposed for 24h to either medium alone,  $\beta$ -glucan as a positive control, or different concentrations of oxidized LDL (A-B), acetylated LDL (C-D) or native LDL (A-B). After washing and six days of resting period, monocytes were re-stimulated with Pam3Cys (n=6, \*  $p < 0.05$ , \*\*  $p < 0.01$  compared to the untrained control).



**Supplementary Figure III.** Effect of continuous exposure of monocytes to oxLDL during 7 days on cytokine production. Adherent monocytes were first exposed for 24h to RPMI,  $\beta$ -glucan or 10  $\mu$ g/ml oxLDL, where after the stimulus was washed away and the cells were rested in RPMI medium supplemented with pooled human serum with or without 5  $\mu$ g/ml oxLDL for 6 days. After 6 days, the cells were re-stimulated with LPS and Pam3Cys, and IL-6 and TNF $\alpha$  levels were measured in the supernatant. (n=6, \* p<0.05 compared to the untrained control)

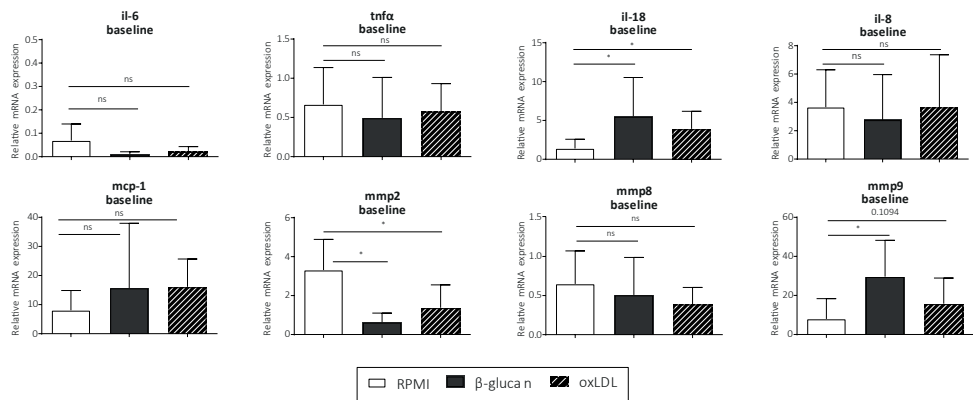


**Supplementary figure IV.** Characteristics of trained cells on day 6. The monocyte-derived trained cells on day 6 were studied for differentiation markers for M1 and M2, as well as DC markers, and compared to the untrained control. No differences were observed in M2 markers ARG1 and CD163, or M1 markers iNOS, TLR4 and TLR2 or DC markers HLA-DRB1, CD83 and DC-SIGN ( $n \geq 3$  per group).

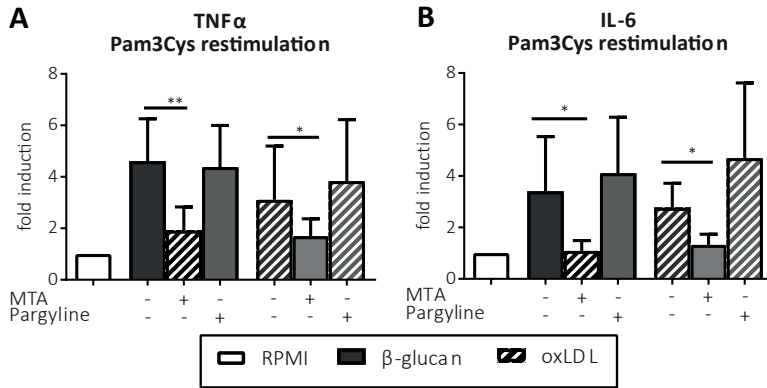




**Supplementary Figure V.** baseline mRNA expression levels. RNA expression levels of unstimulated cells were measured at day 6 and compared to the untrained control. No significant differences were observed for il-6, tnfa, il-8 and mmp8 expression levels. Baseline expression of il-18 and mmp9 were significantly increased, and a non-significant trend was observed for mcp-1. Mmp2 expression was significantly downregulated upon training (n=6, \* P<0.05).



**Supplementary Figure VI.** Methyltransferase inhibition prevents training of monocytes by oxLDL. TNF $\alpha$  (A) and IL-6 (B) levels were measured in supernatants of adherent monocytes primed for 24h with either  $\beta$ -glucan as a positive control, oxLDL or medium alone in the absence or presence of the histone methyltransferase inhibitor MTA and the histone demethylase inhibitor Pargyline. At day 6, cells were re-stimulated with Pam3Cys. Results are shown as fold induction, compared to the untrained control group. (n=8 per group, \* P<0.05, \*\* p<0.01)





## PART II

# TRAINED IMMUNITY AND ATHEROSCLEROSIS IN VIVO



# CHAPTER 5

## OXIDIZED PHOSPHOLIPIDS ON LIPOPROTEIN(A) ELICIT ARTERIAL WALL INFLAMMATION AND AN INFLAMMATORY MONOCYTE RESPONSE IN HUMANS

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## ABSTRACT

### Background

Elevated lipoprotein(a) [Lp(a)] is a prevalent, independent cardiovascular risk factor but the underlying mechanisms responsible for its pathogenicity are poorly defined. Since Lp(a) is the prominent carrier of pro-inflammatory oxidized phospholipids (OxPL), part of its atherothrombosis might be mediated through this pathway.

### Methods

In vivo imaging techniques MR imaging, <sup>18</sup>F-FDG-PET/CT and SPECT/CT were used to measure subsequently atherosclerotic burden, arterial wall inflammation and monocyte trafficking to the arterial wall. Ex vivo analysis of monocytes was performed using FACS analysis, inflammatory stimulation assays and transendothelial migration assays. In vitro studies to the pathophysiology of Lp(a) on monocytes were performed using an in vitro model for trained immunity.

### Results

We show that subjects with elevated Lp(a) (108 [50-195] mg/dL; n=30) have increased arterial inflammation and enhanced PBMCs trafficking to the arterial wall, compared with subjects with normal Lp(a) (7 [2-28] mg/dL; n=30). In addition, monocytes isolated from subjects with elevated Lp(a) remain in a long-lasting primed state, as evidenced by an increased capacity to transmigrate and produce pro-inflammatory cytokines upon stimulation (n=15). In vitro studies show that Lp(a) contains OxPL and augments the pro-inflammatory response in monocytes derived from healthy controls (n=6). This effect was markedly attenuated by inactivating OxPL on Lp(a) or removing OxPL on apo(a).

### Conclusions

These findings demonstrate that Lp(a) induces monocyte trafficking to the arterial wall and mediates pro-inflammatory responses through its OxPL content. These findings provide a novel mechanism by which Lp(a) mediates cardiovascular disease.

## INTRODUCTION

Lipoprotein(a) [Lp(a)] is a plasma lipoprotein composed of apolipoprotein(a) [apo(a)] covalently bound to apolipoprotein B-100 (apoB) of a low density lipoproteins (LDL)-like particle (Figure S1). A role for Lp(a) in atherogenesis has now been established by both meta-analyses of epidemiological studies, as well as by genome-wide association and Mendelian randomization studies showing a strong, independent and likely causal relationship between Lp(a) and myocardial infarction, stroke, peripheral artery disease and calcific aortic valve stenosis.<sup>1,2</sup>

Despite evidence of causality and the fact that one-fifth of the general community has elevated Lp(a) levels of >50 mg/dl<sup>3</sup>, Lp(a) is not routinely measured in clinical risk assessment. This reluctance appears to be due to a lack of understanding of the pathophysiological mechanisms by which Lp(a) mediates cardiovascular disease (CVD), as well as the lack of specific therapeutic interventions to potentially lower Lp(a) levels<sup>4</sup>. Consistent with known mechanisms of atherogenicity of LDL, Lp(a)'s atherogenic capacity could in part reflect the response-to-retention phenomenon of an apoB-containing particle<sup>5</sup>. The disproportionately large impact of Lp(a) on CVD risk compared with LDL, however, implies that additional pathogenic pathways need to be considered<sup>6</sup>.

Over the last decade, a large body of experimental and clinical evidence has documented that Lp(a) is the main lipoprotein carrier of phosphocholine (PC) containing oxidized phospholipids (OxPL) in plasma<sup>7-9</sup>, which has led to the hypothesis that a major component of the risk mediated by Lp(a) is through its content of OxPL<sup>10</sup>. OxPL is recognized as a danger associated molecular pattern (DAMP) by pattern recognition receptors (PRRs) on innate immune cells, leading to a wide range of pro-inflammatory and plaque destabilizing processes<sup>11-13</sup>. In epidemiological studies, the level of OxPL on apoB-containing lipoproteins (OxPL-apoB), which predominantly reflects Lp(a) associated OxPL, was found to be highly predictive of future CVD risk<sup>14,15</sup>. Most recently, a further link between Lp(a), OxPL and inflammation was discovered, showing that the risk of Lp(a) and OxPL-apoB in mediating coronary atherogenesis and cardiovascular events was conditional on an inflammatory IL-1 haplotype<sup>16</sup>. Here, we report that subjects with elevated plasma Lp(a) levels are characterized by increased inflammatory activity of the arterial wall as well as an enhanced inflammatory response of circulating monocytes, for which OxPL carried by Lp(a) were identified as obligatory intermediates. These findings indicate a novel link between Lp(a), its associated OxPL and accelerated atherogenesis in humans.



## METHODS

### Study subjects

This was a single-center study in subjects with elevated Lp(a) and subjects with normal Lp(a), matched for age, gender and BMI. Exclusion criteria consisted of active smoking, history of clinically-manifest cardiovascular disease or diabetes, and lipid lowering therapies including statins. Each subject provided written informed consent. The study and radiation exposure (<10mSv in total) was approved by the local institutional review board and conducted according to the principles of the International Conference on Harmonization–Good Clinical Practice guidelines.

### Biochemical Measurements

EDTA plasma obtained through venous blood samples were taken after overnight fasting and stored using standardized protocols. Plasma Lp(a), total cholesterol (TChol), high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were analyzed using commercially available enzymatic methods. Low density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald equation and subsequently corrected for Lp(a)-cholesterol ( $LDLc - [Lp(a) \text{ in mmol/L} \times 0.3]$ )<sup>17</sup>. Oxidized phospholipids on apolipoprotein B-100 (OxPL-apoB) and on apolipoprotein(a) (OxPL-apo(a)), were measured as follows: apoB-100 and apo(a) were captured on microtiter wells using appropriate antibodies and the content of OxPL quantified by monoclonal antibody E06 and reported as nM, as previously described<sup>10</sup>.

### MR imaging

MR images were obtained with a 3.0 T whole-body scanner (Ingenia, Philips Medical Systems, Best, The Netherlands), using an 8 channel dedicated bilateral carotid artery coil (Shanghai Chenguang Medical Technologies, Shanghai, China). The normalized wall index (NWI= mean wall area/outer wall area) was calculated<sup>18</sup> by one blinded experienced reader at the core laboratory using semi-automated measurement software (VesselMass, Leiden, the Netherlands).

### PET/CT imaging

PET/CT scans were performed on a dedicated scanner (Philips, Best, the Netherlands)<sup>19</sup>. All subjects fasted for at least 6 hours prior to infusion of <sup>18</sup>F-FDG (200 MBq). After 90 min, subjects underwent PET imaging initiated with a low-dose non-contrast enhanced CT for attenuation correction and anatomic co-registration. PET/CT images were analyzed by blinded readers using OsiriX (Geneva, Switzerland; <http://www.osirix-viewer.com/>). The target-to-background-ratio (TBR) was calculated from the ratio of arterial SUV and venous background

activity, as described previously<sup>19</sup>.

### SPECT/CT imaging

SPECT/CT scans were performed at 3, 4.5 and 6 hours post infusion of <sup>99m</sup>Tc-labeled autologous peripheral blood mononuclear cells (PBMCs) (200 MBq) on a dedicated scanner (Symbia T16, Siemens, Erlangen, Germany)<sup>20</sup>. In each subject, around 20·10<sup>6</sup> PBMCs were isolated and labeled with <sup>99m</sup>Tc-(1100 megabecquerel/2milliliter). SPECT/CT images were analyzed by blinded readers using OsiriX<sup>20</sup>. Accumulation of labeled PBMCs in the arterial wall was quantified by the ratio of the averaged maximum counts in the artery divided by the averaged mean counts in the blood, reported as the arterial-wall-to-blood-ratio (ABR)<sup>20</sup>.

### Flow cytometry

Blood samples were collected into K3EDTA BD Vacutainer1 (BD Biosciences, San Jose, CA) tubes, after which 50 µL of whole blood was added to each flow cytometry tube plus the appropriate amounts of each antigen specific mAb were added, defined after titration of the dose recommended by the manufactures. See supplementary for detailed information.

### Cells

Human PBMCs were isolated from blood using Ficoll-Paque Premium (d=1.077g/ml) density gradient centrifugation (GE Healthcare, UK). For in vitro experiments, human PBMCs were isolated from blood of healthy volunteers (Sanquin Bloodbank, Nijmegen, The Netherlands). CD14<sup>pos</sup> monocytes were isolated using a Ficoll-Paque plus (Pharmacia Biotech, Uppsala, Sweden) density gradient, followed by magnetic activated cell sorting (MACS) using CD14-coated MicroBeads according to manufacturer's instructions (MACS, Miltenyi Biotec, Leiden, The Netherlands). Primary Human Arterial Endothelial Cells (HAECs) were purchased from Lonza (Baltimore, MD) and routinely cultured on fibronectin (FN; 10 µg/ml) coated culture flasks (TPP, Switzerland) or glass-slides for imaging in EGM2 medium containing SingleQuots (Lonza). Endothelial cells were cultured up to 6 passages.

### Antibodies and reagents

Actin (clone AC-40) mAb was purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). Filamentous actin (F-actin) was stained with Phalloidin Texas-red (Invitrogen), and the nucleus with Hoechst 33258. β-glucan (b-1,3-(D)-glucan) was kindly provided by Professor David Williams (College of Medicine, Johnson City, USA), r-apo(a) (8K-IV), r-apo(a) 17K and 17KΔLBS10 were derived as previously described<sup>9</sup>. Stimuli and inhibitors used are oxPAPC (Invivogen), LPS (Sigma-Aldrich, St. Louis, MO; from E. coli serotype O55:B5, further purified as described)<sup>21</sup>, Pam3Cys (EMC microcollections, Tübingen, Germany; L2000), anti-OxPL

antibody Mouse monoclonal EO6 (Avanti Polar Lipids, Inc, Alabaster, Alabama, USA, 330001), purified mouse IgM Isotype control antibody (Biolegend, San Diego, California, USA, 401602). In the monocyte assays, Lp(a), LDL and HDL were isolated from 3.5 mL plasma from subjects with either high or normal Lp(a) levels by KBr-density gradient ultracentrifugation; plasma density was adjusted to  $d=1.25$  g/mL with solid KBr (0.385 g/mL plasma), a discontinuous gradient was formed by carefully layering 2 mL of  $d=1.225$  g/mL KBr solution, followed by 4 mL of  $d=1.100$  g/mL KBr solution, and a top layer of 3 mL of  $d=1.006$  g/mL KBr solution was added. The samples were centrifuged for 20 hours at  $10^{\circ}\text{C}$  at 29,000 rpm in a SW 41 Ti rotor (Beckman Coulter Inc., CA). The LDL, Lp(a) and HDL fractions were sliced out and dialyzed against PBS. LAL assay showed negligible endotoxin levels ( $<0.05$  EU/mL) in the isolated fractions. For the western immunoblots, Lp(a) was purified from the lipid apheresis eluate of a single donor undergoing LDL apheresis with high Lp(a) levels (140 mg/dL) and 16 KIV and 18 KIV repeats. The apheresis eluate was subjected to ultracentrifugation in NaBr and the  $1.08 > d > 1.06$  g/mL fraction was collected. This fraction was applied to a SW-400 gel filtration column, which was eluted in 0.5 mL fractions. Each fraction was assayed for the presence of apo(a) and apoA-I by ELISA. The fractions containing apo(a) but not apoA-I were pooled, buffer exchanged into PBS and concentrated using Amicon centrifugal filter units (Millipore). Apo(a) was dissociated from Lp(a) based on the methods described previously<sup>22,23</sup>. Briefly, the solution containing Lp(a) was adjusted to a final concentration of 100 mM ACA and 2 mM dithiothreitol and incubated at room temperature for 1 h. The reaction mixture was adjusted to a density of 1.3 g/mL with NaBr and the solution was centrifuged at 50,000 rpm to separate apo(a) from the LDL released by reduction and intact Lp(a).

### **Western immunoblot**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of apo(a), Lp(a), and LDL (100 ng protein), in reducing (with beta-mercaptoethanol (BME)) and non-reducing conditions, was carried out using 3-8% gradient polyacrylamide gels in Tris-acetate SDS running buffer. Proteins were transferred to a PVDF membrane, which was blocked with 3% BSA in PBS-Tween. Membranes were then incubated with either biotinylated LPA4 (0.004 mg/mL), biotinylated EO6 (0.004 mg/mL), or MB47 (0.006 mg/mL) probing for apo(a), OxPL, and human apoB-100 respectively. Protein detection was performed with streptavidin conjugated to horseradish peroxidase (HRP) from (R and D Systems) and visualized with ECL (Amersham).

### **Detection of OxPL by LC-MS/MS**

To document the presence of PC-containing OxPL in Lp(a) purified by ultracentrifugation, Lp(a) was isolated from 3 donors. Details of the LC-MS/MS procedures is described in the Supplement.

### Transendothelial migration assay

Human aortic endothelial cells (HAECs) were cultured on a fibronectin (FN)-coated glass cover to confluency and stimulated overnight, i.e. 12 hours with TNF $\alpha$  (10 ng/ml)<sup>24</sup>. Monocytes ( $1 \times 10^6$  cells/ml) were added to the HAECs monolayer for 30 min in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C and fixed with 3.7% formaldehyde (Sigma-Aldrich, Zwijndrecht, the Netherlands). After fixation, multiple images were recorded with a Zeiss Axiovert 200 microscope (Plan-apochromat 10x/0.45 M27 Zeiss-objective; Carl Zeiss Inc., Jena, Germany), and analyzed using Image-J software (<http://rsb.info.nih.gov/nih-image/>). After treatment and fixation in 3.7% formaldehyde in PBS (+1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>), cells were permeabilized in PBS-T (PBS + 0.1% Triton-X100) for 10 min, stained for nuclei and filamentous actin for 45 min, washed and stored in PBS (+1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>) until imaged using a confocal laser-scanning microscope (LSM510 META, Carl Zeiss MicroImaging, Jena, Germany) with a Zeiss Plan-Apochromat, 63x/1.40 oil objective. Monocyte motility was determined by plating freshly isolated monocytes into fibronectin (Sanquin Reagents, Netherlands) coated Lab-Tek Chambers (Thermo-Scientific, Rochester, NY) containing N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES)-buffer + 1mM CaCl<sub>2</sub> + 0.5% v/v human albumin and 0.1% w/v glucose. Videos were acquired using a wide-field microscope (Axio Observer Z1, Carl Zeiss MicroImaging, Jena, Germany) equipped with a humidified atmosphere climate chamber with 5 % CO<sub>2</sub> and 37 °C, and analysed using the Tracking Tool (Gradientech, Uppsala Science Park, Uppsala, Sweden).

### In vitro monocyte priming and challenge assay

Monocytes were isolated from PBMCs by adhering  $5 \cdot 10^6$  PBMCs/ml to polystyrene for 1h at 37°C, 5% CO<sub>2</sub> in 96-, or 6-well culture plates (Corning, New York, USA). Non-adherent cells were removed by washing three times with warm PBS. Monocytes were cultured in RPMI 1640 Dutch-modified culture medium (Life Technologies/Invitrogen, Breda, The Netherlands) supplemented with 10 mM glutamine (Invitrogen), 10  $\mu$ g/mL gentamicin (Centraform), 10 mM pyruvate (Invitrogen) and 10% pooled human plasma. The monocytes were primed for 24 hours with either RPMI,  $\beta$ -glucan (5  $\mu$ g/ml), isolated Lp(a) (0.5-250  $\mu$ g/ml), LDL (10  $\mu$ g/ml), HDL (10  $\mu$ g/ml), r-apo(a) constructs (8K-IV 0.001-0.5  $\mu$ g/ml; 17K and 17K $\Delta$ LBS10 0.1  $\mu$ g/ml), OxPAPC (0.05-10  $\mu$ g/ml) or 10% plasma from subjects with high or normal Lp(a) levels (before and after apoB precipitation of the apoB-containing lipoprotein fraction with polyethylene glycol 8000, Sigma P-2139<sup>25</sup>), after which the cells were washed and incubated in supplemented culture medium for 6 days. In inhibition experiments using E06 (1 nM) or IgM control (1 nM), stimuli were pre-incubated with the inhibitors 1h prior to adding the stimuli to the cells. After 6 days of rest in supplemented RPMI medium, cells were exposed to either medium alone, 10  $\mu$ g/mL Pam3Cys or 10 ng/ml lipopolysaccharide. After 24h of

incubation, supernatants were collected and stored at -20°C. Production of cytokines was measured in supernatants by ELISA according to the manufacturer's instructions (TNF $\alpha$  (R&D Systems, Minneapolis, USA) and IL-6 (Sanquin, Amsterdam, The Netherlands), or the human inflammatory cytokine Cytometric Bead Array according to the manufacturer's instructions (BD Biosciences, 551811).

### **Statistical Analysis**

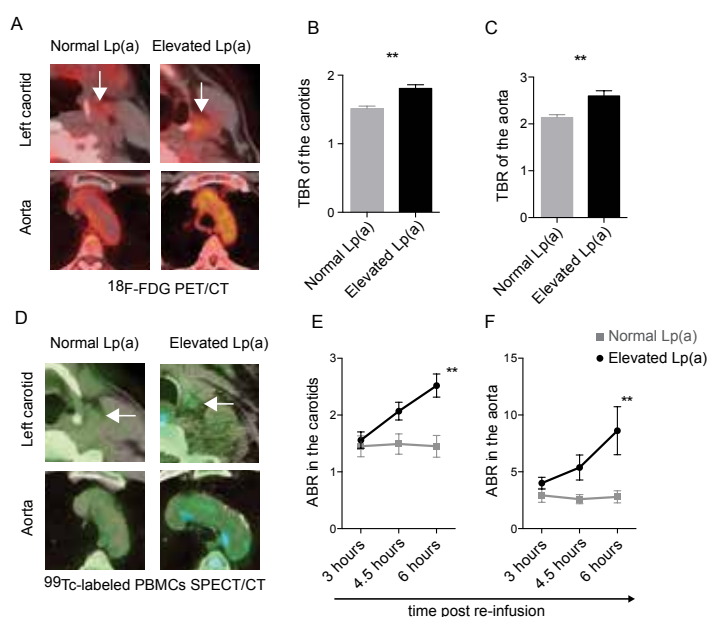
Data are presented as the mean (standard deviation) or the median (min-max) for continuous variables, and as a number (percentage) for categorical variables. To examine the difference in clinical characteristics, imaging parameters and ex vivo monocyte phenotype and function between subjects with elevated or normal Lp(a) we performed student T-test or Mann-Whitney U test for normal and non-normal distributed variables, respectively. Normality was examined by means of inspection of histograms and the Shapiro-Wilk test. Variances in both groups were examined and tested for similarity using Levene's Test for Variance. To assess the differences in PBMCs accumulation as imaged with SPECT, a linear mixed model for repeated measurements was applied (fig 1D-F). In vitro monocyte experiments were performed at least 6 times, and analyzed using the Wilcoxon signed-rank test. A two-sided P-value below 0.05 was considered statistically significant. All data were analyzed using Prism version 5.0 (GraphPad software, La Jolla, California) or SPSS version 21.0 (SPSS Inc., Chicago, Illinois).

## RESULTS

### Increased arterial wall inflammation in subjects with elevated Lp(a)

To determine if Lp(a) influences arterial wall inflammation, we included subjects with elevated Lp(a) levels (mean of 108 mg/dL [range 50-195]; n=30) or normal Lp(a) levels (mean of 7 mg/dL [range 2-28]; n=30) matched for age, gender and body mass index (Table 1). None of the subjects were currently smoking, had a history of clinically-manifest cardiovascular disease or diabetes, or used lipid-lowering therapies such as statins. Aside from Lp(a) levels, subjects did not differ in blood pressure, non-Lp(a) lipoprotein profile or circulating leukocytes (Table 1). As expected, subjects with elevated Lp(a) exhibited significantly higher levels of OxPL-apoB and specifically, OxPL on apo(a) lipoproteins (OxPL-apo(a)) (Table 1). The normalized wall index (NWI) of the carotids, as assessed with magnetic resonance imaging (MRI)<sup>18</sup>, was not significantly different between groups (Table 1, Figure S2A).

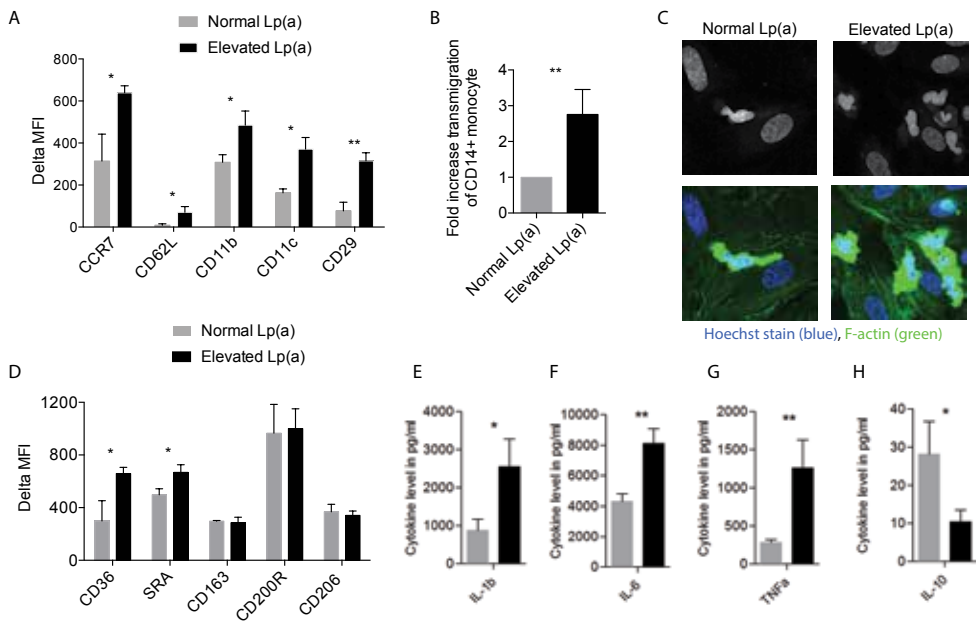
Positron emission tomography with computed tomography (PET/CT) imaging was performed to image arterial wall <sup>18</sup>F-fluorodeoxyglucose uptake (<sup>18</sup>F-FDG), as a marker of arterial wall inflammation<sup>26</sup>. In subjects with elevated Lp(a), arterial wall <sup>18</sup>F-FDG uptake, quantified as



**Figure 1:** Increased arterial wall inflammation in subjects with elevated Lp(a). (A) Cross-sectional <sup>18</sup>F-FDG PET/CT images demonstrating an increased <sup>18</sup>F-FDG uptake (yellow) in the left carotid (top, indicated by white arrow) and aorta (bottom) in a subject with normal Lp(a) (left) and a subject with elevated Lp(a) (right), quantified as the maximum target to background ratio (TBR) in the (B) carotid arteries, and (C) ascending aorta in subjects with elevated Lp(a) (n=30) and normal Lp(a) (n=30), (D) cross-sectional SPECT/CT images demonstrating increased autologous <sup>99m</sup>Tc-labeled PBMCs accumulation (blue; at T=6 hours post-infusion), depicted as the arterial wall to blood pool ratio (ABR) at the level of (E) the carotids and (F) ascending aorta in subjects with elevated Lp(a) (n=15) and normal Lp(a) (n=15). \*\*=p<0.01.

the target to background ratio (TBR), was significantly higher compared with subjects with normal Lp(a) (Figure 1A); both in the carotids (Figure 1B) as well as in the aorta (Figure 1C). Of note, the inflammatory activity in subjects with elevated Lp(a) did not correlate to arterial wall dimensions (NWI, normalized wall index) as assessed with MRI (Figure S2B).

In view of the intricate relation between resident arterial wall macrophages and circulating monocytes<sup>27</sup>, we subsequently investigated the impact of elevated Lp(a) on circulating immune cells in vivo. We used an imaging approach to monitor the trafficking of technetium-99m (<sup>99m</sup>Tc)-labeled autologous PBMCs to the arterial wall, using single photon emission computed tomography with computed tomography (SPECT/CT)<sup>20</sup>. PBMCs were found to accumulate at a substantially higher rate in the arterial wall within 3-6 hours after re-infusion in subjects with elevated Lp(a) compared with those with normal Lp(a) (Figure 1D). The arterial wall-to-blood-pool ratio (ABR)<sup>20</sup> values were significantly higher in subjects with



**Figure 2. Monocytes have an activated and inflammatory phenotype in Lp(a) subjects.** (A) Bar graphs display the expression (quantified as delta MFI) of chemokine, adhesion and transmigration markers on monocytes as assessed with flow cytometry in subjects with elevated Lp(a) ( $n=15$ , black bars) compared with normal Lp(a) ( $n=15$ , grey bars), (B) bar graph and (C) microscope images demonstrating increased endothelial transmigration of monocytes (calculated per at least 4 fields of view) isolated from subjects with elevated Lp(a) ( $n=15$ , black bar) compared with normal Lp(a) ( $n=15$ , grey bar), coinciding with augmented spreading and adhesion as illustrated nucleus (Hoechst, blue) and F-actin (green) stain, (D) bar graphs showing expression (quantified as delta MFI) of scavenger and other receptors on monocytes in subjects with elevated Lp(a) ( $n=15$ , black bars) compared with subjects with normal Lp(a) ( $n=15$ , grey bars), (E-H) in response to an overnight challenge to Pam3Cys ( $10 \mu\text{g/ml}$ ), monocytes isolated from subjects with elevated Lp(a) ( $n=15$ , black bars) produced higher levels of IL-18 (E), IL-6 (F) and TNF $\alpha$  (G) and lower levels of IL-10 (H), compared with monocytes of subjects with normal Lp(a) ( $n=15$ , grey bars). ^= $p<0.06$ , \*= $p<0.05$ , \*\*= $p<0.01$ , \*\*\*= $p<0.001$ .

elevated Lp(a) in both the carotids (Figure 1E) and aorta (Figure 1F). Collectively, these in vivo imaging studies indicate that subjects with elevated Lp(a) levels have increased arterial inflammatory activity, which coincides with more autologous immune cell accumulation in their arterial wall. Further, there is a strong correlation between the TBR indices from PET/CT imaging and the ABR values from PMBCs imaging and respective Lp(a) plasma levels (Table 2).

### **Monocytes increasingly migrate in subjects with elevated Lp(a)**

We next questioned whether the increased arterial wall immune cell accumulation was preceded by an intrinsic cellular activation leading to enhanced migration. To this end, we analyzed adhesion and migration markers of blood monocytes in subjects with elevated versus normal Lp(a) using flow cytometry (gating strategy in Figure S3A)<sup>28</sup>. Monocytes from subjects with elevated Lp(a) expressed more C-C chemokine receptor type 7 (CCR7), L-selectin (also described as the cluster of differentiation 62 ligand; CD62L) and integrin alpha and beta chains; CD11b, CD11c and CD29<sup>29</sup> (Figure 2A), for which the latter two showed a (borderline) significant correlation with the Lp(a) plasma levels (Table 2).

To evaluate the functional relevance of increased expression of adhesion and migration markers, we performed transendothelial migration (TEM) experiments<sup>24</sup> with monocytes isolated from subjects with and without elevated Lp(a). Twelve hours prior to performing the migration assay, human arterial endothelial cells (HAECs) were stimulated with TNF $\alpha$  to mimic dysfunctional endothelium. Freshly isolated monocytes of subjects with elevated Lp(a) showed a 3-fold higher TEM rate compared with monocytes isolated from subjects with normal Lp(a) (Figure 2B). Staining of the nucleus and F-actin illustrated that monocytes derived from subjects with elevated Lp(a) have augmented spreading and adhesion compared with monocytes of subjects with normal Lp(a) (Figure 2C). In addition, monocytes of subjects with elevated Lp(a) also showed higher motility when plated on fibronectin-coated surfaces (movie S1), lending further support to an increased trans migratory tendency.

Thus, in subjects with elevated Lp(a), circulating monocytes exhibit an enhanced capacity to adhere and transmigrate the endothelium. Whereas generalized markers of inflammation, such as plasma levels of CRP, do not relate with Lp(a) levels, a number of inflammatory markers measured on the monocytes of the subjects are proportional to their Lp(a) levels (Table 2).

### **Atherogenic monocyte response in subjects with elevated Lp(a)**

In addition to migration markers, monocytes of subjects with elevated Lp(a) expressed increased levels of the scavenger receptors CD36 and SRA (Figure 2D), also correlating to Lp(a) plasma levels (Table 2), whereas the expression of other receptors such as CD163, CD200R and CD206 was not different (Figure 2D). To assess whether this activated phenotype also



translated to an enhanced inflammatory response, we performed a challenge assay using Toll-like receptor (TLR) ligands. After an overnight stimulation with a TLR2 ligand (Pam3Cys), monocytes isolated from subjects with elevated Lp(a) produced higher levels of tumor necrosis factor alpha (TNF $\alpha$ ), interleukin 1 beta (IL-1 $\beta$ ) and IL-6 (Figure 2E-G). Conversely, the production of IL-10 was lower (Figure 2H). Similar results were observed upon stimulation with the TLR4 ligand lipopolysaccharide (LPS) (Figure S4A-D). In aggregate, we observed an activated state of monocytes, potentially resulting from their priming by Lp(a), or some associated component found in the plasma of subject with elevated Lp(a).

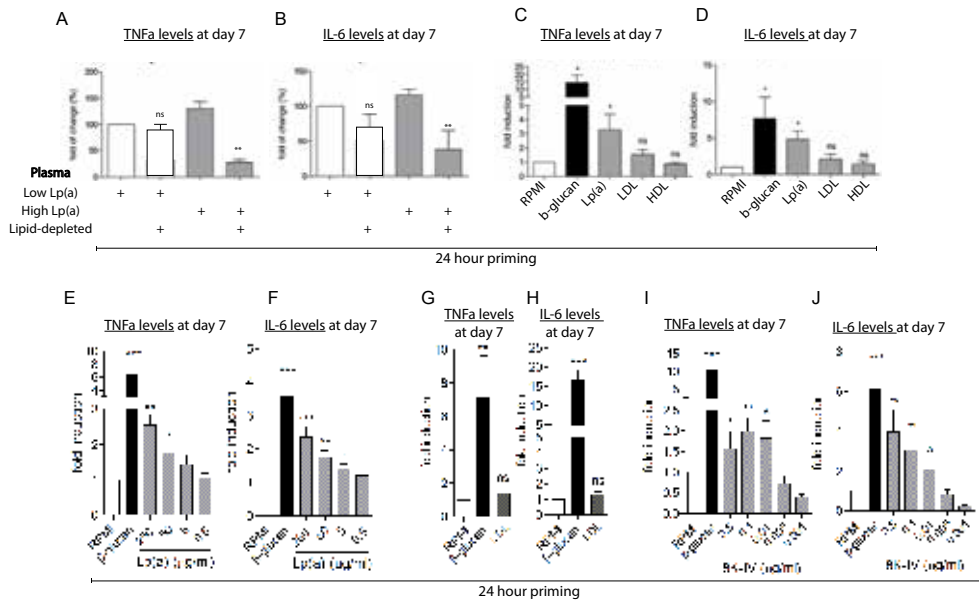


Figure 3. Isolated Lp(a) primes monocytes towards a responsive state. (A,B) priming of healthy monocytes with plasma of a subject with high Lp(a) (1st grey bar) for 24h resulted in an increased production of TNF $\alpha$  and IL-6 upon re-stimulation with Pam3Cys (10  $\mu$ g/mL) on day 6, compared with plasma of normal Lp(a) (1st white bar). In addition, after lipid-depletion (2nd white and grey bar) this effect of high Lp(a) plasma was profoundly reduced compared to the undepleted control (n=6), (C,D) priming of healthy monocytes with  $\beta$ -glucan (5  $\mu$ g/mL, positive control, black bar) or Lp(a) (grey bar, 250  $\mu$ g/mL) for 24h, induced an increased production of TNF $\alpha$  and IL-6 upon re-stimulation with Pam3Cys (10  $\mu$ g/mL) on day 6, compared with priming with RPMI (negative control, white bar). LDL (grey bar, 10  $\mu$ g/mL) or HDL (grey bar, 10  $\mu$ g/mL) did not induce this increase (n=6) (E,F) in addition, various concentrations of Lp(a) (grey bars) for 24h, resulted in a dose-dependent increased production of TNF $\alpha$  and IL-6 upon re-stimulation with Pam3Cys (10  $\mu$ g/mL) on day 6 (n=6) compared to priming with RPMI (negative control, white bar) (G,H) priming of healthy monocytes with LDL (10  $\mu$ g/mL) (grey bar) did not result in higher cytokine levels after Pam3Cys (10  $\mu$ g/mL) compared to the negative control RPMI (white bar), in contrast to  $\beta$ -glucan (5  $\mu$ g/mL, positive control, black bar) (n=6), whereas (I,J) priming of healthy monocytes with  $\beta$ -glucan (5  $\mu$ g/mL, positive control, black bar) or the r-apo(a) construct 8K-IV (grey bars) induced increased cytokine levels after Pam3Cys (10  $\mu$ g/mL) (n=6), compared with RPMI (negative control, white bar). \*= $p$ <0.05, \*\*= $p$ <0.01, \*\*\*= $p$ <0.001.

**Lp(a) and its associated OxPL induce prolonged responsiveness in monocytes**

Lp(a) was isolated by ultracentrifugation, the lipid soluble material was extracted, and LC-MS/MS was performed on the extracted material and shown to have the following OxPLs: POVPC, PGPC, PONPC, PAzPC and KDdiA-PC, with PONPC being the most abundant (Figure S5A-B).

To address priming by Lp(a), or some associated component found in the plasma of subject with elevated Lp(a), we performed a series of in vitro studies, in which we analyzed the ability of various plasma components to activate monocytes. As a positive control for monocyte activation we used  $\beta$ -glucan, a cell wall component of *C. Albicans*, since we have extensively demonstrated that  $\beta$ -glucan induces a long-lasting pro-inflammatory phenotype in monocytes<sup>30</sup>.

First, priming of healthy monocytes with plasma from subjects with high Lp(a) for 24 hours, with a subsequent washout and resting period, increased the production of TNF $\alpha$  and IL-6 after an overnight challenge with the TLR2 and TLR4 ligands Pam3Cys or LPS, respectively (Figure 3A,B and Figure S6A,B), whereas normal Lp(a) plasma did not activate monocytes. Lipid depletion of the above plasma profoundly decreased the cytokine production of the in-origin high Lp(a) plasma, indicating that a lipophilic particle might be responsible for the observed effect (Figure 3A,B and Figure S6A,B).

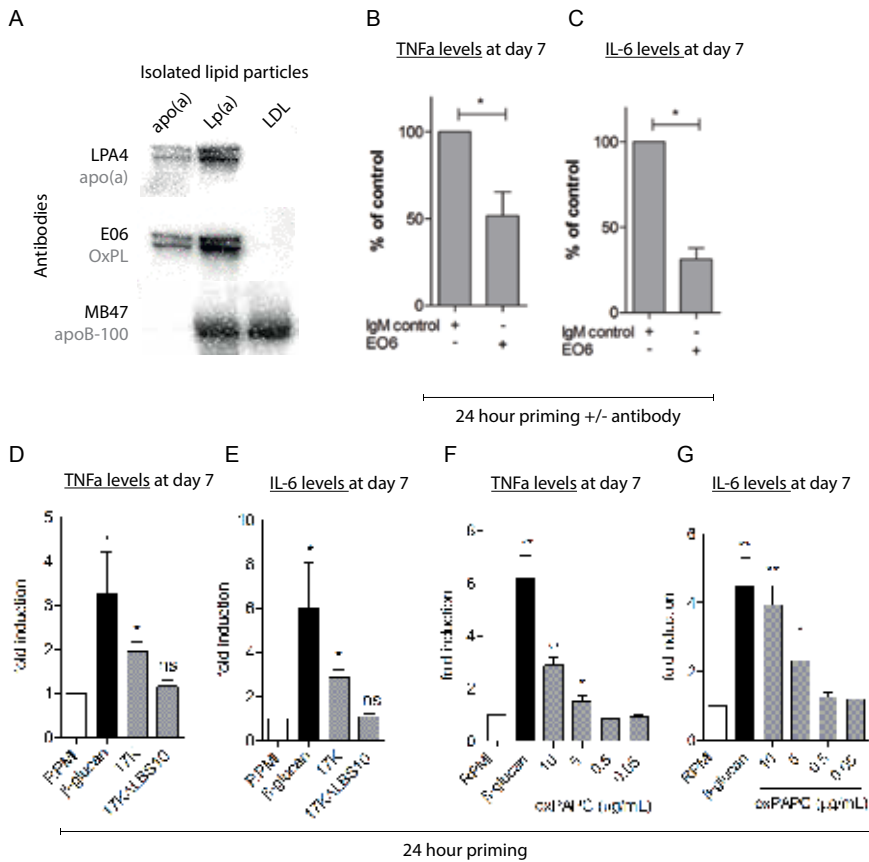
In line with the difference in Lp(a) levels between both plasma, priming of healthy monocytes with purified Lp(a) for 24 hours, induced an increased production of TNF $\alpha$  and IL-6 at day 7, after an overnight TLR challenge (Figure 3C-F and Figure S6C-F). Please note that priming with the isolated lipoprotein fractions LDL and HDL did not induce monocyte activation (Figure 3C,D and Figure S6C,D).

Next, we assessed the effects of Lp(a)'s main components separately; using isolated LDL, apo(a) separated from Lp(a), and recombinant apo(a) [r-apo(a)] constructs containing various numbers of kringle<sup>9</sup>. Whereas priming with LDL had no prolonged effects on monocytes (Figure 3G,H and Figure S6G,H), priming with an 8 kringle IV (8K-IV) r-apo(a) construct elicited an enhanced cytokine response in healthy donor monocytes at day 7 after overnight stimulation with TLR ligands (Figure 3I,J and Figure S6I,J).

In view of the role of Lp(a) as carrier of OxPL<sup>7-9</sup>, we hypothesized an important role for OxPL in monocyte activation. In support of this concept, we show via immunoblotting that both Lp(a) and apo(a) contain OxPL, whereas LDL isolated from the same plasma as Lp(a) does not (Figure 4A). To further demonstrate the role of OxPL in mediating the activation of monocytes, we utilized monoclonal antibody E06, which binds to the PC moiety of OxPL and potently inhibits their pro-inflammatory properties, but does not bind to native, non-oxidized phospholipids<sup>31-33</sup>. Whereas the 8K-IV apo(a) construct, containing covalently bound OxPL<sup>9</sup>, potently activated monocytes (Figure 3I,J and Figure S6I,J), pre-incubation of the 8K-

IV construct with E06 inhibited its priming effects (Figure 4B,C and Figure S6K,L).

The role of OxPL was further validated using 2 additional constructs consisting of 17 kringles (17K) but varying in the functionality of the lysine binding site (LBS) on kringle IV type 10 (KIV<sub>10</sub>), which in turn is related to the ability of the apo(a) constructs to have covalently bound OxPL<sup>9,34</sup>. Thus, 17K is a r-apo(a) construct with an intact LBS that has covalently bound OxPL, whereas 17KΔLBS10 has a mutated LBS and lacks immunologically detected OxPL<sup>9,34</sup>. Consistent with our previous findings with Lp(a), priming with 17K induced a state of



**Figure 4. OxPL induce the enhanced monocyte response . (A)** Immunoblotting of purified apo(a), Lp(a) and LDL; OxPL, apo(a), and apoB-100 resolved on SDS-PAGE in reducing and non-reducing conditions, were detected using the monoclonal antibodies E06, LPA4 and MB47 respectively. E06 immunostained apo(a) and Lp(a) but not LDL isolated from the same subject, (B,C) pre-treatment with the E06 antibody (1 nM) against OxPL inhibited the increased monocyte response after priming with 8K-IV (0.1 μg/mL, grey bars) and subsequent challenge with Pam3Cys, shown as % of the initial priming with 8K-IV, corrected for IgM control antibody (n=6), (D,E) priming with β-glucan (5 μg/mL, positive control, black bar) and the r-apo(a) construct 17K (0.1 μg/mL, grey bar) induced increased cytokine production after Pam3Cys (10 μg/mL), whereas 17KΔLBS lacking OxPL (0.1 μg/mL, grey bar) did not (n=6), (F,G) priming with β-glucan (5 μg/mL positive control, black bar) and oxPAPC (grey bars) increased the monocyte responsiveness compared with RPMI (negative control, white bar) (n=6). ^=p<0.06, \*=p<0.05, \*\*=p<0.01, \*\*\*=p<0.001.

increased responsiveness in monocytes, whereas such effects were absent with the mutant 17KΔLBS10 (Figure 4D,E and Figure S6M,N). Finally, we show that short-term priming with purified oxidized-1-palmitoyl-2-arachidonoyl-sn-3-glycero-phosphocholine (OxPAPC), a mixture of OxPL, also results in a dose-dependent enhanced cytokine production at day 7 in response to an overnight challenge with TLR ligands (Figure 4F,G and Figure S6O,P). Collectively, these data show that subjects with elevated Lp(a) exhibit increased arterial wall inflammation coinciding with an increased responsiveness of monocytes, in which OxPL carried by Lp(a) are obligatory mediators.

## DISCUSSION

In this translational work, we provide evidence in support of the concept that pro-inflammatory OxPL are major contributors to the atherogenicity of Lp(a) by showing that (i) subjects with elevated Lp(a) have increased arterial inflammation *in vivo*, (ii) monocytes isolated from subjects with elevated Lp(a) remain in a long-lasting activated state *ex vivo*, and (iii) Lp(a) elicits the pro-inflammatory response in healthy monocytes *in vitro*, an effect markedly attenuated by removing or inactivating OxPL present on Lp(a) and specifically on apo(a).

We demonstrate an increased inflammatory activity in the arterial wall in subjects with elevated Lp(a), who present with enhanced  $^{18}\text{F}$ -FDG uptake in the arterial wall proportional to their Lp(a) levels. In analogy to the enhanced glucose consumption in inflamed tissue, arterial wall  $^{18}\text{F}$ -FDG uptake has been proposed as a marker of atherogenic inflammation, supported by previous studies documenting  $^{18}\text{F}$ -FDG uptake primarily in macrophage-rich areas in atherosclerotic plaques<sup>26</sup>. In addition, we report that this increased local inflammatory activity coincides with a higher accumulation rate of autologous PBMCs in the arterial wall as visualized by SPECT/CT imaging. Whereas we previously demonstrated augmented influx of PBMCs in advanced atherosclerotic plaques<sup>20</sup>, the present finding emphasizes that in subjects at increased risk for atherosclerotic disease due to Lp(a) elevation, PBMC accumulation in the arterial wall is significantly enhanced also at sites without plaques. The increased arterial inflammatory signal in our subjects with elevated Lp(a) cannot be attributed to differences in atherosclerotic burden for two reasons: first, the normalized wall index of the carotids on MRI did not differ from controls, and second, inflammatory measures of the arterial wall did not correlate with structural arterial wall metrics. The absence of a consistent relation between Lp(a) elevation and established surrogate markers of atherosclerotic burden observed in this as well as in previous studies<sup>35,36</sup> implies that Lp(a) may increase CVD risk by enhancing plaque vulnerability rather than accelerating plaque burden.

To address whether intrinsic cellular activation precedes enhanced PBMC migration in Lp(a) subjects, we subsequently analyzed isolated circulating monocytes, which are key contributors to the technetium uptake by PBMCs<sup>20</sup>. Using *ex vivo* assays, we were able to recapitulate our *in vivo* findings. First, we found that monocytes isolated from subjects with elevated Lp(a) displayed an increased capacity to transmigrate the endothelium. Second, we observed that monocytes isolated from subjects with elevated Lp(a) have enhanced capacity to produce pro-inflammatory cytokines upon TLR stimulation. Previous experimental studies already showed that the apo(a) component of Lp(a) was able to also activate endothelial cells<sup>37</sup>, whereas apo(a) also co-localized with the adhesion molecule Mac-1 in the arterial

wall<sup>38</sup>. When integrating these data, subjects with elevated Lp(a) might be burdened by a double hit comprising activated monocytes as well as endothelial cells, both predisposing for increased arterial wall inflammation.

In support of a causal role for Lp(a) in driving monocyte activation, a series of in vitro experiments substantiate that (i) high Lp(a) plasma induce monocyte activation in vitro, which is abolished after lipid-depletion, (ii) among the isolated lipoprotein fractions, only Lp(a) and apo(a) (particles both carrying OxPL) have the capacity to induce long-term monocyte activation.

In humans, more than 85% of plasma lipoprotein-associated OxPL are present on Lp(a). On Lp(a), OxPL is present in the lipid phase (i.e. removable from Lp(a) by delipidation with organic solvents) as well as covalently attached to apo(a)<sup>8,9</sup>. Here we found five oxPL species on Lp(a), comprising POVPC, PGPC, PONPC, PAzPC and KDdIA-PC, with PONPC being the most abundant. Previously, PONPC was shown to be the most abundant OxPL in the debris of distal protection devices from coronary bypass graft, carotid and renal interventions.<sup>39</sup>

Recombinant 17K apo(a) containing covalently bound OxPL but not 17KΔLBS r-apo(a), which lacks E06 detectable OxPL<sup>9</sup>, reproduced Lp(a) priming of monocyte activation. The key epitope that E06 recognizes is the phosphocholine (PC) headgroup when presented in the proper conformation on OxPL. Sequencing the V<sub>H</sub> and V<sub>L</sub> chains of E06 revealed that these were identical to those regions of the T15 IgA natural antibody, which has been crystallized with its antigen, the PC moiety<sup>40</sup>. Further detailed lipidomic study of a large variety of lipids, oxidized lipids and oxidized-lipid adducts revealed that like T15, the IgM E06 only bound to oxidized phosphocholine containing phospholipids (e.g. PC- and only when the sn2 side chain was oxidized and or adducted to a protein), but not to native PAPC suggesting that oxidation products of the sn2 PUFA generated from oxidized PAPC appeared to generate the correct conformational epitope of the PC to allow recognition by E06<sup>32,40</sup>.

Here, we demonstrate that the proinflammatory effects of apo(a) can be blocked by E06. Further we show that a recombinant apo(a) that contains bound OxPL is capable of activating monocytes, whereas the nearly identical, but mutated recombinant apo(a) that has lost the ability to bind OxPL does not have monocyte activation properties, indicating the necessity for OxPL to mediate monocyte activation. In aggregate, these data indicate that the OxPL carried by Lp(a) are obligatory danger signals in eliciting the prolonged potentiation of the monocyte response in vitro.

These findings are also complementary to prior reports demonstrating that OxPL are danger associated molecular patterns (DAMPs). The PC headgroup on OxPL is recognized by multiple

innate PRRs<sup>12</sup>; for example, both CD36 and SR-B1 bind the PC on OxPL<sup>31,41</sup> and consequently, E06 blocked the uptake of OxLDL by these macrophage scavengers<sup>42</sup>. In addition, both acute and chronic lung injury in mice have been shown to lead to the generation of OxPL in the brochiolavage fluid, which in turn caused cytokine production by macrophages that was dependent on TLR4, and E06 was capable of blocking the pro-inflammatory properties of the OxPL<sup>43</sup>. Similarly, the OxPL content of apoptotic cells was shown to induce endothelial expression of IL-8, and more recently, the OxPL on apo(a) was shown to be necessary for apo(a) mediated upregulation of IL-8 by macrophages<sup>33,34</sup>. Hence, the mechanisms by which OxPL accelerates atherosclerosis have been attributed both to mediating uptake of OxLDL into macrophages to generate foam cells, as well as to a variety of pro-inflammatory and plaque destabilizing processes<sup>11–13</sup>. In addition, recent studies suggest that such DAMP/PAMPs induce long-term potentiation of their inflammatory response *in vitro* via epigenetic alterations<sup>30,44,45</sup>. Future studies will need to focus on elucidating how and for how long OxPL-Lp(a) induce adaptive responses of monocytes.

Whereas the present study cannot substantiate that OxPL also is a prerequisite for monocyte activation in humans *in vivo*, it is interesting to note that our subjects with elevated Lp(a) exhibited 4-fold higher OxPL-apoB levels (OxPL associated with all apoB lipoproteins) and even 20-fold higher OxPL-apo(a) levels (OxPL associated with Lp(a) lipoproteins) compared with subjects with normal Lp(a). This increased circulating OxPL content in subjects with elevated Lp(a) suggests that a comparable impact of OxPL may hold true *in vivo*. In support, we have previously shown that OxPL-apoB levels were potent predictors of progressive atherosclerosis as well as the risk of cardiovascular disease and death in patients<sup>14–16,46</sup>.

It remains to be established whether lowering of Lp(a) and OxPL will also lead to a reduction in arterial wall inflammation and eventually CVD risk. Recently, an antisense-based approach was reported that specifically lowered Lp(a) levels by ~80%, and to a similar degree their associated OxPL-apoB and OxPL-apo(a) levels<sup>47,48</sup>. With the development of such therapies to effectively lower Lp(a)<sup>49</sup>, we should be able to design appropriate clinical trials to aid in dissecting the (causal) relation between Lp(a) and cellular as well as arterial wall inflammation. In addition to Lp(a)-lowering therapies, the present findings may also provide novel targets to modulate the atherogenic impact of Lp(a). Because the OxPL content of Lp(a) appears to mediate the pro-inflammatory effect on monocytes, oxidation-specific epitope targeted therapy using specific antibodies may also bear clinical potential<sup>50</sup>. At present these approaches are being evaluated in experimental settings only<sup>51,52</sup>.

In summary, our findings strengthen the case for the inflammatory hypothesis of Lp(a) by addressing the increased inflammatory activity in the arterial wall, corresponding to an enhanced accumulation of activated immune cells, in which Lp(a)'s associated OxPL are obligatory intermediates.



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## TABLES

**Table 1.** Clinical characteristics of included subjects

Characteristic	Subjects with normal Lp(a) (n=30)	Subjects with elevated Lp(a) (n=30)	P value
Age, y	53 ± 12	52 ± 11	0.858
Gender, %male (n)	45 (9)	43 (15)	0.820
BMI, kg/m <sup>2</sup>	24 ± 4	24 ± 3	0.914
DBP, mmHg	79 ± 7	81 ± 8	0.399
SBP, mmHg	131 ± 8	134 ± 12	0.144
Smoking, %active	0 (0)	0 (0)	-
NWI	0.42 ± 0.06	0.39 ± 0.04	0.161
Lp(a), mg/dL	7 [2-28]	108 [50-195]	<0.001
OxPL-apo(a), nM	3.0 [0.5-26.0]	69.1 [40.9-92.5]	<0.001
OxPL-apoB, nM	4.1 [2.3-6.3]	15.8 [5.9-31.1]	<0.001
TChol, mmol/L	5.21 ± 0.83	5.79 ± 1.44	0.127
LDLc*, mmol/L	2.91 ± 0.80	2.80 ± 1.16	0.621
HDLc, mmol/L	1.68 ± 0.42	1.60 ± 0.40	0.481
TG, mmol/L	0.80 [0.24-2.18]	0.82 [0.39-2.16]	0.684
CRP, mg/L	2.30 [0.40-4.40]	1.13 [0.30-1.90]	0.180
HbA1c, mmol/mol	36 [34-39]	35 [33-38]	0.100
Creatinine, umol/L	76 [66-80]	70 [55-87]	0.304
ALT, U/L	27 ± 10	27 ± 6	0.766
AST, U/L	25 ± 11	27 ± 8	0.427
WBC, 10 <sup>9</sup> /L	5.4 ± 1.3	5.7 ± 1.3	0.522
Monocytes, 10 <sup>9</sup> /L	0.43 ± 0.15	0.43 ± 0.12	0.996
Neutrophils, 10 <sup>9</sup> /L	3.1 ± 1.4	3.3 ± 1.09	0.710
Lymphocytes, 10 <sup>9</sup> /L	2.4 ± 2.6	2.2 ± 1.7	0.666
Eosinophils, 10 <sup>9</sup> /L	0.16 ± 0.13	0.15 ± 0.16	0.891
Basophils, 10 <sup>9</sup> /L	0.03 ± 0.01	0.03 ± 0.01	0.809

Data are presented as mean ± SD, n (%) or median [min-max]. ALT indicates alanine transaminase; apo(a), apolipoprotein(a); apoB, apolipoprotein B-100; AST, aspartate

transaminase; BMI, body mass index; CRP, C-reactive protein, CVD, cardiovascular disease (including myocardial infarction, stroke or peripheral artery disease); HbA1c, hemoglobin A1c; HDLc, high density lipoprotein cholesterol; DBP, diastolic blood pressure; LDLc, low density lipoprotein cholesterol; Lp(a), lipoprotein(a); NWI, normalized wall index; OxPL, oxidized phospholipids; SBP, systolic blood pressure; Tchol, total cholesterol; TG, triglycerides; WBC, white blood cell count, \*LDLc corrected for Lp(a)-cholesterol.

**Table 2.** Correlations between inflammatory metrics and Lp(a) levels

Method	Parameter	Spearman correlation with Lp(a) level
PET imaging	TBR, carotid	0.31 <sup>^</sup>
	TBR, aorta	0.45 <sup>**</sup>
SPECT imaging	ABR, carotid	0.72 <sup>*</sup>
	ABR, aorta	0.65 <sup>*</sup>
Whole plasma level	CRP, mg/L	0.24
Flow cytometric assay	CD11b, ΔMFI	0.27
	CD11c, ΔMFI	0.42 <sup>^</sup>
	CD29, ΔMFI	0.50 <sup>*</sup>
	SRA, ΔMFI	0.42 <sup>^</sup>
	CD36, ΔMFI	0.48 <sup>*</sup>
Ex vivo monocyte cytokine production	TNFα, pg/mL	0.27
	IL-6, pg/mL	0.18

Correlation coefficients of the Spearman's rho test are shown, correlations are flagged with <sup>^</sup>p<0.06, <sup>\*</sup>p<0.05, <sup>\*\*</sup>p<0.01, <sup>\*\*\*</sup>p<0.001.

TBR and ABR are imaging metrics, CRP was measured in whole plasma, TNFα and IL-6 were measured as products of monocytes after TLR ligand challenge, and CD11b, CD11c, CD29 and CD36 expression on monocytes were measured by flow cytometry as described in Methods.



## SUPPLEMENTAL MATERIAL

### Additional Methods

#### Flow cytometry

The following fluorochrome labeled mAb were used: phycoerythrin-CyChrome7 (PE-CyTM7)-anti CD14, allophycocyanin (APC)-Cy7-anti CD16, peridinin-chlorophyll-protein (PerCP)-Cy5.5-anti HLA-DR, PE-anti CD11b, APC-anti CD11c, APC-anti CD18, APC-anti CD29, fluorescein isothiocyanate (FITC)-anti CD32, APC-anti CD36, PE-anti CD62L, APC-anti CD192 (CCR2), FITC-anti CD195 (CCR5) [all from BD Biosciences, San Jos, CA, USA]; PE-anti CD204 (SR-A) [R&D systems, Abingdon, UK] and PE-anti CD197 (CCR7) [Biolegend, SanDiego, CA, USA]. Samples were incubated for 20 min at RT in the dark. To lyse red blood cells, 700  $\mu$ L of PharmLyse1 (BD Biosciences, San Jose, CA) was added for 12 min, after which the samples were centrifuged at 400g for 5 min, the supernatant discarded and the samples resuspended in 400  $\mu$ L of staining buffer. All data were collected on a FACS Calibur (Becton Dickinson, NJ), compensation was performed using single color fluorochromes and BD FacsComp beads, and samples were analyzed using FlowJo software (version 5.4+); for gating strategy and representative histograms see Figure S3. Delta mean fluorescence intensity (MFI) was obtained by subtracting median MFI from the isotype from the median MFI of the marker in corresponding color. Please note that the delta MFI values of CD62L were scaled down by a factor 10.000 to fit the bar graph.

#### Lipid Extraction

The lipids were extracted by the method previously described by Folch et al. with modifications.<sup>1</sup>  
<sup>2</sup> The lipid extracts were stored in CM (2:1), flushed with nitrogen, capped and stored at -80°C till analysis. The internal standard mixture spiked into each sample prior to extraction consisted of 1,2-dinonanoyl-sn-glycero-3-phosphocholine (DNPC), 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (17:0 LPC)<sup>3</sup>. Synthetic 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC), and 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL). 1-palmitoyl-2-(4-keto-dodec-3-ene-diyl) phosphatidylcholine (KDdiA-PC) were from Cayman Chemicals (Ann Arbor, MI)

#### HPLC

The detection of oxidized PCs were carried out in reverse-phase (RP) chromatography as reported previously<sup>2, 4-7</sup>. Briefly lipid extracts were reconstituted in an RP solvent system

consisting of Acetonitrile-Isopropanol-Water (65:30:5 vol/vol/vol). Thirty microliters of the sample was injected on to Ascentis Express C18 HPLC column (15cmx2.1mm, 2.7 $\mu$ m; Supelco Analytical, Bellefonte, Pennsylvania, USA) and using a Prominence UFLC system from Shimadzu Corporation (Canby, Oregon, USA). Elution was performed by linear gradient of solvent A (Acetonitrile/Water, 60:40 vol/vol) and solvent B (Isopropanol/Acetonitrile, 90:10, vol/vol) both the solvents containing 10 mM ammonium formate and 0.1% formic acid. The time program used was as follows: initial solvent B at 32% until 4.00 min; switched to 45% B; 5.00 min 52% B; 8.00 min 58% B; 11.00 min 66% B; 14.00 min 70% B; 18.00 min 75% B; 21.00 min 97% B; 25.00 min 97% B; 25.10 min 32% B until the elution was stopped at 30.10 min. A flow rate of 260  $\mu$ l/min was used for analysis, and the column and sample tray were held at 45 and 4°C, respectively.

### Mass Spectrometry

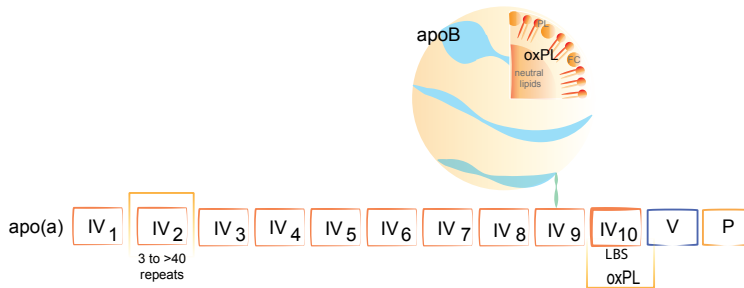
The HPLC system was coupled to 4000 QTRAP® triple quadrupole mass spectrometer system with a Turbo V electrospray ion source from AB Sciex (Framingham, Massachusetts, USA). For phosphatidylcholine, the detection was carried out in positive ion mode by MRM of 6 transitions using PC-specific product ion (184.3 m/z, Da), which corresponds to the cleaved phosphatidylcholine. The MRM settings were as follows: declustering potential=125, entrance potential=10, collision energy=53, collision cell exit potential=9 and dwell time= 50 msec.<sup>8,9</sup>

For each synthetic OxPL, POVPC, PGPC, PONPC, PAzPC and KDdiA-PC external calibration (i.e., using plasma-free standard solutions) was carried out to estimate linear range and sensitivity for these analytes and relative ionization compared to DNPC at equimolar levels. Concentrations of analytes were determined from calibration curves (1/x weighted linear regression) plotted as ratio of analyte peak area/DNPC peak area versus the amount of analyte on a column. The instrument was tuned by direct infusion of poly(propylene glycol) (PPG) in both positive and negative modes and external mass calibration was performed at regular intervals. Retention time window in MRM was set to detect peaks of significance within 60 seconds of confirmed retention time and data was collected utilizing Analyst® Software 1.6 (AB Sciex). Multi-quant® Software 2.1 (AB Sciex) was used to compare peak areas of internal standards and unknown analytes to quantitate the results.

## SUPPLEMENTAL FIGURES

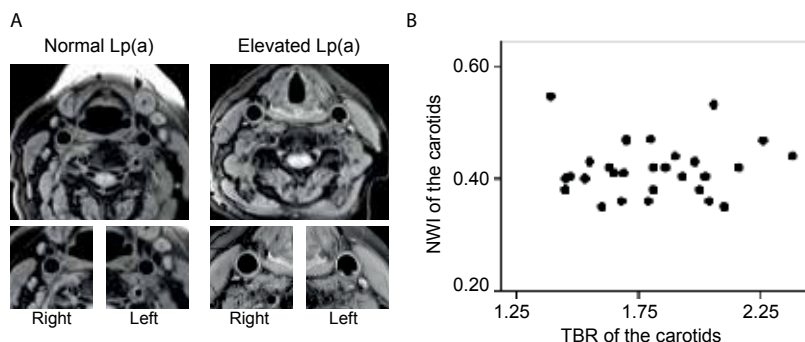
**Fig. S1. Schematic of lipoprotein(a)**

Lipoprotein (a) [Lp(a)] is composed of apolipoprotein(a) [apo(a)] covalently linked to apolipoprotein B-100 (apoB) of LDL. This LDL contains a central core of neutral lipids, surface phospholipids (PL) and free cholesterol (FC). Apo(a) comprises an inactive protease domain (P) and a series of loop structures termed kringle (K), including KIV type 2, (KIV<sub>2</sub>) which is present in 3 to >40 copies. Genes encoding an apo(a) allele with smaller numbers of KIV<sub>2</sub> repeats lead to higher levels of Lp(a) in plasma, presumably due to higher rates of hepatic synthesis. Phosphocholine containing oxidized phospholipids (OxPL) are present in the lipid phase and covalently bound to apo(a) in the areas near KIV-10 and KV.



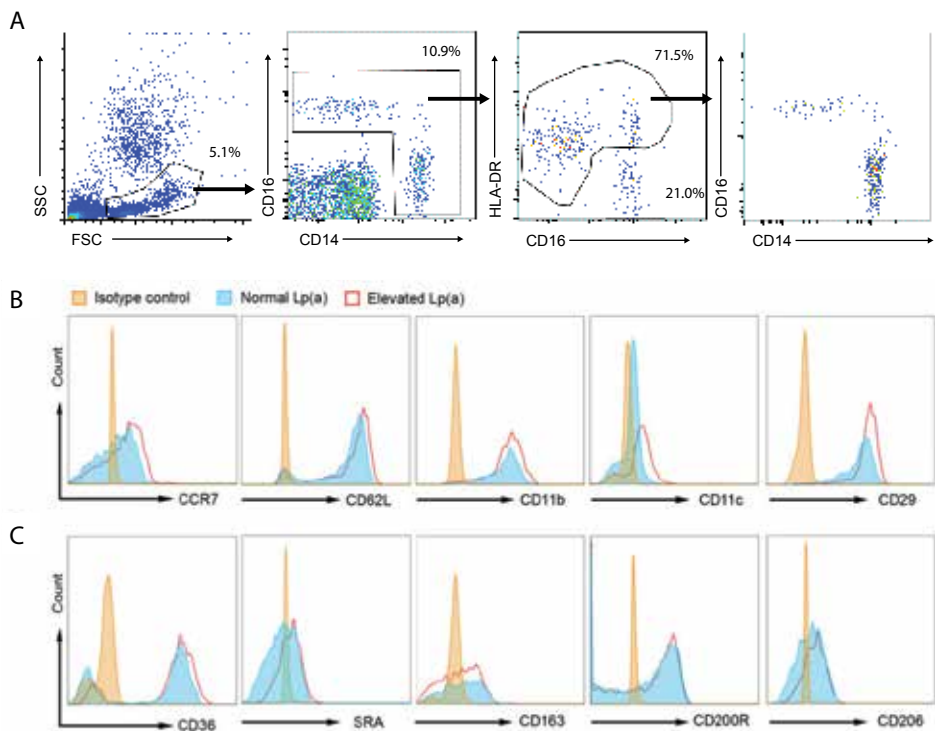
**Fig. S2. Arterial wall dimensions assessed with MR imaging**

(A) Cross-sectional MR images used to quantify the arterial wall dimension, expressed as the normalized wall index (NWI), of the carotid arteries of normal Lp(a) and elevated Lp(a) subjects, (B) scatterplot illustrating no correlation between the arterial wall dimension (NWI, normalized wall index) and inflammation (TBR, target to background ratio, as assessed with  $^{18}\text{F}$ -FDG PET/CT) in subjects with elevated Lp(a).



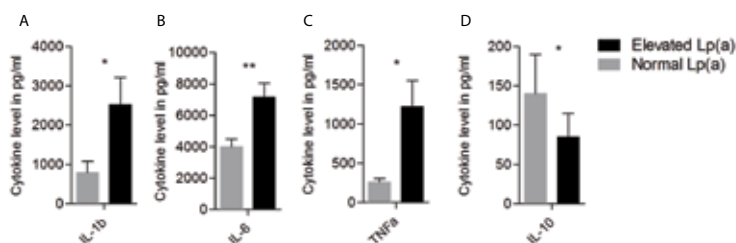
**Fig. S3. Gating strategy to define monocyte population and representative histograms**

(A) The monocyte population was gated as follows; the leukocyte population was gated on a forward side scatter and subsequently viewed on a CD14/CD16 plot in which the CD14<sup>pos</sup>CD16<sup>pos</sup> cell cloud was gated, after which these cells were then shown on the CD16/HLA-DR plot to exclude the HLA-DR<sup>neg</sup>CD16<sup>pos</sup> cells. For gating graphs log scale was used, percentages of cells in gates are shown. Cells were analyzed using FlowJo software (version 5.4+). (B) Representative FACS histograms (of Figure 2A) show the expression of chemokine, adhesion and transmigration markers and (C) the expression of scavenger and other receptors (Figure 2D) on monocytes as assessed by flow cytometry of subjects with elevated Lp(a) (red line) compared with subjects with normal Lp(a) (blue line). The isotype control (orange line) was similar between the two groups and therefore only one isotype control is showed.



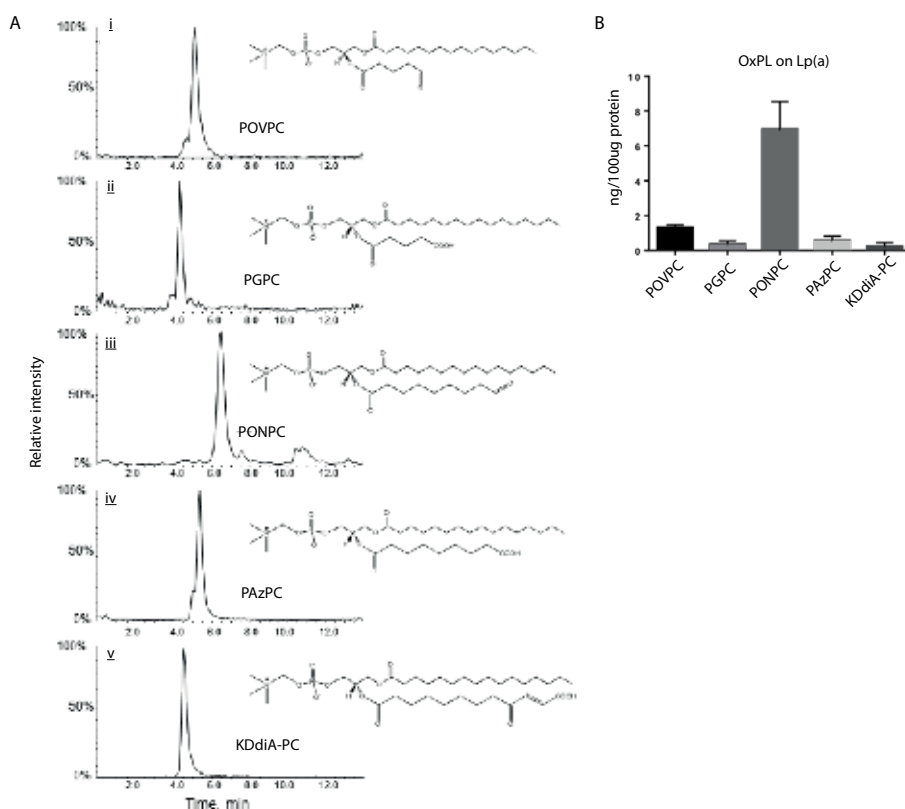
**Fig. S4. Monocytes of Lp(a) subjects have an increased response to LPS**

In response to an overnight challenge to LPS (10 ng/ml) monocytes isolated from subjects with elevated Lp(a) (black bars, n=15) produced higher levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 and lower levels of IL-10, compared to monocytes of subjects with normal Lp(a) (grey bars, n=15). \*= $p$ <0.05, \*\*= $p$ <0.01.



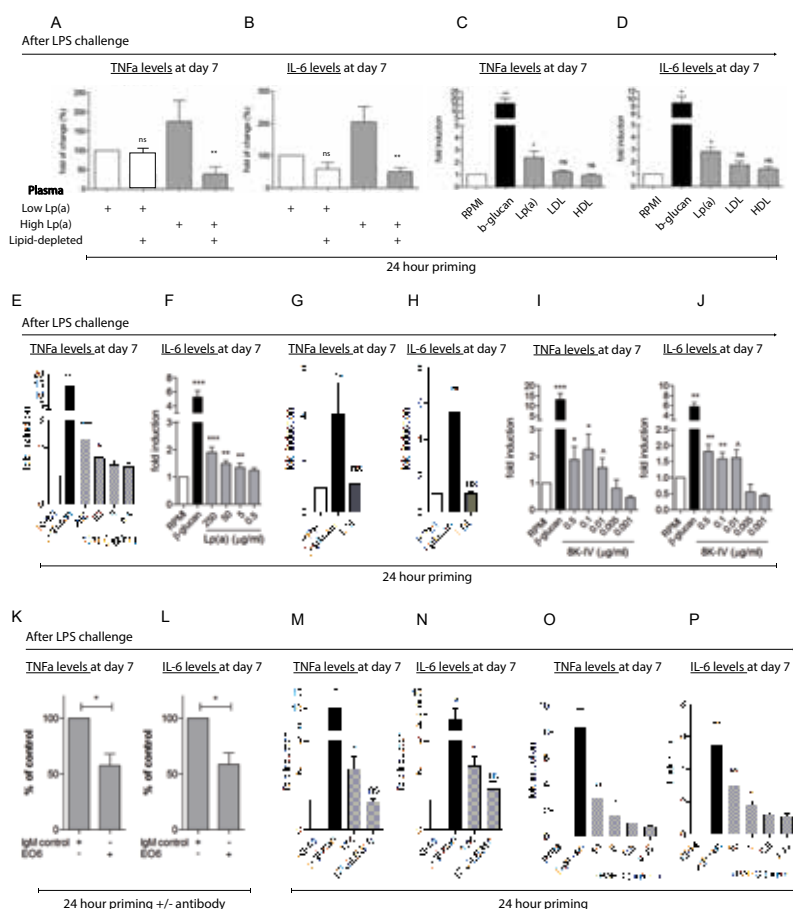
**Fig. S5. Levels of OxPC molecules identified in human Lp(a)**

Quantitation was based on internal standard DNPC, details are given in methods. (A) Identification of oxPC molecules on human Lp(a). Single ion plots of MRM analysis of human Lp(a) lipid extract. Reversephase separation of the most abundant fragmented OxPC products (i)POVPC, (ii) PGPC, (iii) PONPC, (iv) PAzPC, (v) KDdiA-PC (n=3), and (B) quantification of oxPC molecules on human Lp(a) (n=3).

**Fig. S6. Lp(a) and its OxPL load induce an enhanced monocyte response to LPS**

(A,B) priming healthy monocytes with plasma with high Lp(a) (grey bars) for 24h resulted in an increased production of TNF $\alpha$  and IL-6 upon re-stimulation with LPS (10 ng/mL) on day 6, compared with plasma of normal Lp(a) (white bars). In addition, after lipid-depletion this effect was profoundly reduced (n=3), (C,D) priming of healthy monocytes with  $\beta$ -glucan (5  $\mu$ g/mL, positive control, black bar) or Lp(a) (grey bar, 250  $\mu$ g/mL) for 24h, induced an increased production of TNF $\alpha$  and IL-6 upon re-stimulation with LPS (10 ng/mL) on day 6, compared with priming with RPMI (negative control, white bar), LDL (grey bar, 10  $\mu$ g/mL) or HDL (grey bar, 10  $\mu$ g/mL) (n=6), (E,F)

in addition we show a dose-dependent increased production of TNF $\alpha$  and IL-6 after LP(a) priming, upon re-stimulation with LPS (10 ng/mL) on day 6, compared with priming with RPMI (negative control, white bar) (n=6), (G,H) priming with LDL (10  $\mu$ g/mL, grey bar) did not increase cytokine production after LPS (10 ng/mL) (n=6), (I,J) priming of healthy monocytes with  $\beta$ -glucan (5  $\mu$ g/mL, positive control, black bar) or the r-apo(a) construct, 8K-IV, (grey bars) increased TNF $\alpha$  and IL-6 production after LPS (10 ng/mL) (n=6), compared to RPMI (negative control, white bar), (K,L) pre-treatment with the E06 antibody (1 nM) against OxPL inhibited the priming effects of 8K-IV (0.1  $\mu$ g/mL, grey bars), whereas IgM (1 nM) did not (n=6), (M,N)  $\beta$ -glucan (5  $\mu$ g/mL, positive control, black bar) and the r-apo(a) construct 17K priming (0.1  $\mu$ g/mL grey bar) increased cytokine production upon LPS (10 ng/mL), whereas 17K $\Delta$ LBS lacking OxPL (0.1  $\mu$ g/mL, grey bar) did not (n=6), (O,P) priming with  $\beta$ -glucan (5  $\mu$ g/mL, positive control, black bar) and oxPAPC (grey bars) increased the monocyte response upon LPS (10 ng/mL) compared to RPMI (negative control, white bar) (n=6).  $\wedge$ =p<0.06, \*=p<0.05, \*\*=p<0.01, \*\*\*=p<0.001





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# CHAPTER 6

INNATE IMMUNE CELL ACTIVATION AND EPIGENETIC  
REMODELING IN SYMPTOMATIC AND ASYMPTOMATIC  
ATHEROSCLEROSIS IN HUMANS IN VIVO

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**Atherosclerosis (2016) 254:228-236**

## ABSTRACT

### Background and aims

We have recently reported that monocytes can undergo functional and transcriptional reprogramming towards a long-term pro-inflammatory phenotype after brief in vitro exposure to atherogenic stimuli such as oxidized LDL. This process is termed 'trained immunity', and is mediated by epigenetic remodeling and a metabolic switch towards increased aerobic glycolysis. We hypothesize that trained immunity contributes to atherogenesis. Therefore, we investigated the inflammatory phenotype and epigenetic remodeling of monocytes from patients with and without established atherosclerosis.

### Methods

Monocytes were isolated from 20 patients with severe symptomatic coronary atherosclerosis (total plaque score >4 on coronary computed tomography angiography) and 17 patients with asymptomatic carotid atherosclerosis and matched controls for both groups. Ex vivo stimulation, RNA analysis and Chromatin Immunoprecipitation was performed.

### Results

Monocytes from patients with symptomatic atherosclerosis have a higher production of pro-inflammatory cytokines upon LPS stimulation than healthy controls (TNF $\alpha$  499 $\pm$ 102 versus 267 $\pm$ 45 pg/ml,  $p=0.01$ ). This was associated with lower histone 3 lysine 4 trimethylation (H3K4me3) (19% vs 33%,  $p=0.002$ ), and lower H3K27me3 (0.005% vs 0.8%,  $p<0.0001$ ) on the TNF $\alpha$  promoter. Furthermore, relative mRNA expression of the glycolytic rate limiting enzymes hexokinase 2 and phospho-fructokinase was higher in patients (0.7 $\pm$ 0.2 vs 0.3 $\pm$ 0.1 resp. 1.7 $\pm$ 0.2 vs 1.0 $\pm$ 0.1,  $P=0.007$  resp. 0.003) compared to control individuals. Interestingly, this pro-inflammatory phenotype was only present in patients with symptomatic atherosclerosis, and not in patients with asymptomatic carotid atherosclerosis.

### Conclusion

Circulating monocytes of patients with symptomatic, but not asymptomatic, atherosclerosis have a pro-inflammatory phenotype and increased expression of glycolytic enzymes, associated with epigenetic remodeling at the level of histone methylation.

## INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death worldwide, projected to cause 30 million deaths per year in 2030<sup>1</sup>. The main underlying pathological process of CVD is atherosclerosis, a chronic low-grade inflammatory disorder of the arterial wall. Macrophages are the most abundant cells found in plaques and play central roles in the various stages of atherosclerosis<sup>2</sup>. Plaque macrophages are both derived from circulating monocytes that bind to activated endothelial cells and enter the intimal layer as well as from local proliferating plaque macrophages<sup>3</sup>. The mechanism that explains the persistent non-resolving inflammation within atherosclerotic plaques remains to be elucidated.

We recently proposed that trained immunity contributes to the persistent vascular inflammation that characterizes atherosclerotic plaques<sup>4</sup>. Trained immunity denotes the process through which innate immune cells can adopt a long-term pro-inflammatory phenotype after brief exposure to certain pathogen- and danger-associated molecular patterns, such as oxidized low density lipoprotein (oxLDL) particles<sup>5</sup>. Brief in vitro exposure of human monocytes to oxLDL induces a long-lasting pro-inflammatory macrophage phenotype that is characterized by increased pro-atherogenic cytokine and chemokine production and increased foam cell formation. The key mechanisms underlying this phenotype are epigenetic reprogramming at the level of histone methylation and a shift in the intracellular metabolism from oxidative phosphorylation towards an increased aerobic glycolysis (ie. the Warburg effect)<sup>6</sup>.

To test the hypothesis that trained immunity contributes to the development of atherosclerosis, we now performed a first proof-of-concept study in patients with established atherosclerosis. We aimed to investigate whether circulating monocytes of patients with atherosclerosis have a pro-inflammatory phenotype, and whether this is associated with epigenetic marks and change in metabolism that are characteristic of trained immune cells. To explore whether the role of trained immunity differs in the various stages of atherosclerosis, we studied monocytes from patients with symptomatic coronary atherosclerosis and subjects with asymptomatic carotid atherosclerosis.

## MATERIALS AND METHODS

### Study participants

Patients with symptomatic coronary atherosclerosis were selected from patients who had been referred to the cardiac emergency ward of the Canisius Wilhelmina Hospital in Nijmegen from October 2013 to March 2014 because of chest pain, and who were evaluated with coronary computed tomography angiography (CCTA). We selected twenty patients with a total plaque score (TPS)>4 (patients) and twenty control patients with a TPS=0 (controls). Patients and controls were matched for age, gender and body mass index (BMI). Exclusion criteria were an acute coronary syndrome (at present or in the past), presence of a coronary stent, bypass surgery in the past, heart failure liver or renal failure, as well as use of anti-inflammatory medication other than statins, and thrombocyte aggregation inhibitors. Additional exclusion criteria were diabetes, rheumatoid arthritis, or other autoimmune diseases and signs/symptoms of acute infection.

Patients with asymptomatic carotid atherosclerosis were selected using carotid ultrasound from the participants of the population-based IN CONTROL study, from October 2013 to July 2015. A total of 39 asymptomatic subjects were included. Exclusion criteria for this study were a recent cardiovascular event, history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral subcutaneous anti-coagulant therapy, and use of thrombocyte aggregation inhibitors other than acetylsalicylic acid.

All laboratory analyses were performed blinded for group allocation. The study protocol was approved by the Institutional Review Board Arnhem/Nijmegen, The Netherlands, and prospectively registered at Clinicaltrials.gov (number NCT02393768)

### Evaluation of atherosclerotic burden

#### Evaluation of total plaque score (TPS)

CCTA total plaque score was calculated of each patient as described previously<sup>1</sup>. Briefly, a 64-slice MDCT scanner (Philips) was used to obtain CAC and MDCT Image acquisition using an ECG-synchronized axial scan protocol and post-processing CT and CAC studies using IntelliSpace Philips software. Prior to image acquisition, beta-blockers were administered targeting a heart rate of <60 bpm., and patients received nitroglycerin 0.8 mg sublingually. Prospective electrocardiographically gated step-and-shoot contrast-enhanced MDCT imaging was performed, initiated from 10 mm above the level of the left main artery to 10 mm below the inferior myocardial apex with scan parameters being 64 x 0.625 mm sections (2.5 mm), collimation tube currents of 350 to 780 mAs and tube voltage of 100 or 120 kV. In the rare

event that prospective scanning was not possible, retrospective or helix scanning was used. Reconstruction of the MDCT scans was performed with reconstructed images obtained, using an ecg-triggered protocol, at 75% from the previous RR-interval, or at 75% and 40% from the previous RR-interval if a helix scan-protocol was used. The TPS was determined by summing the number of evaluable coronary segments with calcific or non-calcific plaque, or mixed plaque, where non calcified and mixed plaque was assigned with 1 point and calcified plaque with 0 (maximum score = 16). Two independent experienced operators scored all CT images and both operators were blinded for all clinical information.

#### Carotid plaque detection

Carotid ultrasound was performed using an MyLabClass C Ultrasound machine (Esaote BV, Maastricht, The Netherlands) with a 3-11 MHz linear-array transducer. Longitudinal images of both common carotid arteries were obtained in the optimal projection (anterolateral, lateral or posterolateral). Measurement of the intima-medial thickness (IMT) was performed offline by the sonographer at the time of examination, using semi-automatic edge-detection software (Artlab, Esaote BV, Maastricht, The Netherlands) in the far wall. A plaque in the carotid arteries was defined as local thickening of the IMT of >50% compared to the surrounding vessel wall or an IMT > 1.5 mm.

#### **Blood sampling, isolation and stimulation of monocytes**

EDTA plasma was obtained by venous blood sampling and was stored using standardized protocols. Plasma total cholesterol, HDL-c and LDL-c levels were analyzed using commercially available enzymatic methods. PAXgene tubes (Preanalytix, GmbH, Switzerland) were incubated for at least 2h at room temperature before storage at -20°C until RNA isolation. Human peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood using Ficoll-Paque density gradient centrifugation (GE Healthcare, UK). Monocytes were isolated by magnetic activated cell sorting (MACS) using CD14-coated MicroBeads according to manufacturer's instructions (MACS, Miltenyi Biotec, Leiden, The Netherlands). Monocytes were diluted to  $1 \times 10^6$ /ml in RPMI 1640 Dutch-modified culture medium (Life Technologies/Invitrogen, Breda, The Netherlands) supplemented with 10 mM glutamine (Invitrogen), 10 µg/mL gentamicin (Centraform), 10 mM pyruvate (Invitrogen). A total of  $5 \times 10^5$  monocytes were seeded per well on flat-bottom 96-well plates (Corning, New York, USA) and stimulated for 24h with RPMI only as a negative control, 10 ng/ml LPS (Sigma-Aldrich, St. Louis, MO; from E. coli serotype 055:B5, further purified as described<sup>7</sup>) or 10 µg/ml Pam3Cys (EMC microcollections, Tübingen, Germany; L2000). After 24h, plates were centrifuged and supernatants were stored at -20°C until cytokine assessment.



### Cytokine measurements

Cytokine production was determined in supernatants using commercial ELISA kits for IL-1 $\beta$ , TNF $\alpha$  (R&D systems, MN, USA), IL-6 and IL-10 (Sanquin, Amsterdam, The Netherlands) following the instructions of the manufacturer. MCP-1 ELISA was performed following the manufacturers' instructions using antibodies anti-Human CCL2 Purified (14-7099), anti-CCL2 Biotin (13-7096) and Human CCL2 Recombinant Protein (14-8398), all obtained from eBioscience (CA, USA). Cytokines in plasma of patients with coronary atherosclerosis and controls were determined using commercial high sensitive ELISA kits for TNF- $\alpha$  (eBioscience, CA, USA) and IL-6 (R&D systems, MN, USA) and MCP-1 as described above. hsCRP in plasma was determined using the high sensitive ELISA kit for CRP (R&D systems, MN, USA). Cytokines in plasma of subjects with carotid atherosclerosis and controls were measured using the Simple Plex platform (Protein Simple, San Jose, CA).

### RNA isolation from PAXgenes

Total RNA purification was performed according to the manufacturer's instructions (PreAnalytiX GmbH, Switzerland). RNA concentrations were measured using NanoDrop software, and isolated RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-rad, Hercules, CA). qPCR was performed using the SYBR Green method (Applied Biosciences, Carlsbad, CA). Used primers are listed in Supplementary Table 1 (Biolegio, Malden, The Netherlands). Samples were analyzed using the  $\Delta$ Ct method, and HPRT was used as a housekeeping gene.

Gene	Forward primer	Reverse primer
HPRT	CCTGGCGTCGTGATTAGTGAT	AGACGTTTCAGTCTGTCCATAA
IL-6	AACCTGAACCTTCCAAAGATGG	TCTGGCTTGTTCTCTACTACT
TNF $\alpha$	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
MCP-1	CCAGTCACCTGCTGTTATAAC	TGGAATCCTGAACCCACTTCT
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
IL1 $\beta$	CAGCTACGAATCTCCGACCAC	GGCAGGGAACCAGCATCTTC
IL1Ra	GCCTCCGCAGTCACCTAAT	TCCCAGATTCTGAAGGCTTG
STAT3	ACCAGCAGTATAGCCGCTTC	GCCACAATCCGGGCAATCT
HIF1 $\alpha$	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
c-MYC	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
HK2	TTGACCAGGAGATTGACATGGG	CAACCGCATCAGGACCTCA
PFKFB3	ATTGCGGTTTTCGATGCCAC	GCCACAACGTAGGGTCTGT
PDK1	AGTGCCCTCTGGCTGGTTTTG	GCATCTGTCCCCTAACCTC
PKM1	CGAGCCTCAAGTCACTCCAC	GTGAGCAGACCTGCCAGACT

PKM2	ATTATTTGAGGAACTCCGCCGCCT	ATTCCGGGTCACAGCAATGATGG
ASL	CAGTGGACCCCATCATGGAGA	GGCTTTGCTGCCTTGAACATC
MDH1	GGTGCAGCCTTAGATAAAT	AGTCAAGCAACTGAAGTTC
ACAT1	AAGGCAGGCAGTATTGGGTG	ACATCAGTTAGCCCGTCTTTTAC
PDHA1	TGGTAGCATCCCGTAATTTTGC	ATTCGGCGTACAGTCTGCATC
TALDO1	CTCACCCGTGAAGCGTCAG	GTTGGTGGTAGCATCCTGGG

**Table 1. Primers for qPCR**

### Chromatin immunoprecipitation

For the assessment of histone methylation, ChIP was performed as described previously<sup>8</sup>. In short, monocytes were cross-linked in methanol free 1% formaldehyde (28908, Thermo Scientific), followed by sonication to shear-DNA and immunoprecipitation using antibodies against H3K4me3, H3K27me3 and H3K9me3 (Diagenode, Seraing, Belgium). ChIPed DNA was processed further for qPCR analysis. Primers used in the reaction are listed in Supplementary Table 2. Samples were analyzed following a comparative Ct method, myoglobin was used as a negative control for H3K4me3, GAPDH for H3K9me3 and EIF4A2 for H3K27me3, H2B was used as a positive control for H3K4me3, ZNF UTR for H3K9me3 and MYT1 for H3K27me3 according to the manufacturer's instructions.

Promoter of gene	Forward primer	Reverse primer
Myoglobin	AGCATGGTGCCACTGTGCT	GGCTTAATCTCTGCCTCATGAT
H2B	TGTA CTGGTGACGGCCTTA	CATTACAACAAGCGCTCGAC
GAPDH	CACCGTCAAGGCTGAGAACG	ATACCCAAGGGAGCCACACC
ZNF UTR	AAGCACTTTGACAACCGTGA	GGAGGAATTTTGTGGAGCAA
EIF4A2	GGGGAAAGCGAGGTTTAACT	TTACAGGGTCGCTGGAATC
MYT1	TTGGGGTTTGGAATTCTCTG	CAGTCTTTGTTCTCCGCACA
IL6	TCGTGCATGACTTCAGCTTT	GCGCTAAGAAGCAGAACCAC
IL1b	CTGGCGAGCTCAGGTACTTC	ACACATGAACGTAGCCGTCA
TNFa1	AGAGGACCAGCTAAGAGGGA	AGCTTGT CAGGGGATGTGG
TNFa2	CAGGCAGGTTCTCTTCTCT	GCTTTCAGTGCTCATGGTGT
TNFa3	GTGCTTGTTCTCAGCCTCT	ATCACTCCAAAGTG CAGCAG
TNFa4	TGTCTGGCACACAGAAGACA	CCCTGAGGTGTCTGGTTTTC
TNFa5	AGCCAGCTGTTCTCTCTTA	TTAGAGAGAGGTCCCTGGGG
TNFa6	TGATGGTAGGCAGAACTTG	ACTAAGGCCTGTGCTGTTCC

**Table 2. Primers used for ChIP qPCR analysis (5'-3')**

### **Statistics**

Data are presented as mean (standard deviation) for continuous variables and as a number (percentage) for categorical variables. To examine the difference in clinical characteristics between the different patient groups, we performed unpaired student T-tests or Mann-Whitney U tests for normal and non-normal distributed variables, respectively. Ex vivo monocyte experiments were analyzed using the Mann-Whitney U test. In vitro experiments were analyzed using the Wilcoxon signed rank test. A two-sided P-value below 0.05 was considered statistically significant. All data were analyzed using Prism version 5.0 (GraphPad software, La Jolla, California) or SPSS version 21.0 (SPSS Inc., Chicago, Illinois).

## RESULTS

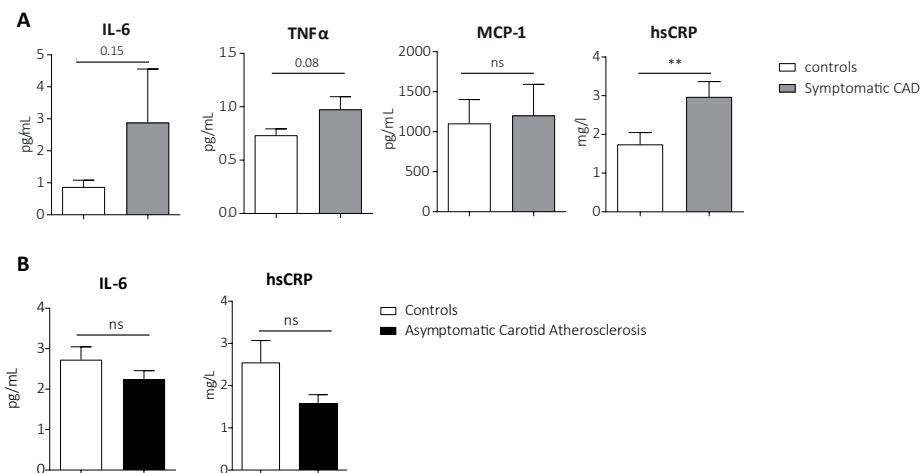
### Baseline characteristics

Baseline characteristics of the patients with symptomatic and subjects with asymptomatic atherosclerosis and controls for both groups without atherosclerosis are listed in Table 1. The average total plaque score (TPS) derived from the Coronary Computed Tomography Angiography (CCTA) was 6.3 in patients (A), indicating severe coronary atherosclerosis, and 0 in controls (B), indicating no coronary atherosclerosis (table 1). Patients with symptomatic coronary atherosclerosis (group A) and controls (group B) were matched for age, gender and body mass index (BMI). There were no significant differences between group A and B in blood pressure, total cholesterol, lipoprotein(a), LDL cholesterol, glucose or plasma creatinine. High density lipoprotein (HDL) cholesterol was slightly lower in patients with coronary artery disease (CAD) (1.45 mmol/L vs. 1.26 mmol/L,  $p=0.04$ ). Statin use was higher in patients than controls (75% vs. 15%,  $p<0.001$ ).

Similarly, there were no significant differences between subjects with (C) and without (D) asymptomatic carotid plaques, besides the presence of plaques measured by carotid ultrasound.

### Increased systemic inflammation in patients with symptomatic atherosclerosis

We measured baseline circulating high-sensitive C-reactive protein (hsCRP), cytokines and chemokines in patients with symptomatic CAD and controls. Plasma samples of patients with

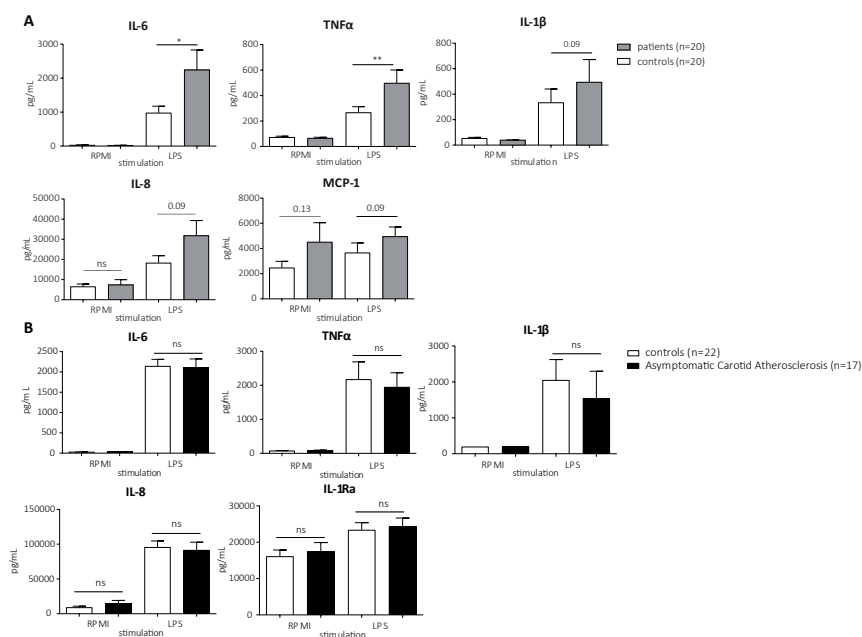


**Figure 1. Inflammatory markers in plasma.** (A). IL-6, TNFα, MCP-1 and hsCRP were measured in EDTA plasma from patients with symptomatic CAD vs. matched controls. Baseline inflammation in patients with symptomatic CAD was increased compared to controls. (B). IL-6 and hsCRP were measured in plasma from patients with asymptomatic carotid atherosclerosis vs. matched controls. Asymptomatic carotid atherosclerosis subjects showed no increased baseline inflammation compared to controls.

symptomatic CAD did not show significantly higher concentrations of IL-6 and TNF $\alpha$  (Fig 1A), although this may be due to the lack of power. There was no difference in MCP-1 concentration. hsCRP was significantly higher in patients compared to controls ( $P<0.01$ ). Subjects with asymptomatic carotid atherosclerosis showed no differences in pro-inflammatory cytokines and hsCRP plasma concentrations (Fig 1B) compared to controls.

### Increased cytokine and chemokine production by monocytes from subjects with symptomatic, but not asymptomatic, atherosclerosis

After overnight stimulation with the Toll-like receptor (TLR) 4 ligand lipopolysaccharide (LPS), monocytes isolated from patients with symptomatic CAD showed an increased production of interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), IL-1 $\beta$ , IL-8 and monocyte chemoattracting protein-1 (MCP-1; Fig 2A) compared to controls. Similar results were observed upon stimulation with the TLR2 ligand Pam3Cys (Supplementary Figure 1). No differences were observed in production of the anti-inflammatory cytokine IL-10, since levels were below



**Figure 2.** Ex vivo stimulation of monocytes (A) Monocytes from patients with symptomatic CAD have a pro-inflammatory phenotype upon ex vivo stimulation of TLR4 compared to matched controls. Monocytes from patients and controls were stimulated with TLR4 ligand LPS for 24h and IL-6, TNF $\alpha$ , IL-1 $\beta$ , IL-8 and MCP-1 were measured in the supernatant after 24h of incubation at 37°C, 5% CO<sub>2</sub> (n=20 vs 20, \*  $p<0.05$ , \*\*  $p<0.01$ ). (B) Monocytes from patients with asymptomatic carotid atherosclerosis do not show an increased pro-inflammatory phenotype upon ex vivo stimulation of TLR4. Monocytes from subjects with carotid atherosclerosis and controls were stimulated with TLR4 ligand LPS for 24h and IL-6, TNF $\alpha$ , IL-1 $\beta$ , IL-8 and MCP-1 were measured in the supernatant after 24h of incubation at 37°C, 5% CO<sub>2</sub>.

detection level (data not shown).

In contrast, monocytes from subjects with asymptomatic carotid atherosclerosis did not show increased production of IL-6, TNF $\alpha$ , IL-1 $\beta$ , IL-8 and MCP-1 upon TLR4 stimulation compared to control subjects without carotid atherosclerosis (Fig 2B). Similar results were obtained after stimulation of the leukocytes by the TLR2 ligand Pam3Cys (Supplementary Figure 2).

## **Circulating cells of patients with symptomatic CAD have a pro-inflammatory phenotype associated with metabolic reprogramming**

### Increased pro-inflammatory cytokine expression

To further characterize the pro-inflammatory phenotype in the patients with symptomatic CAD, we measured baseline RNA expression of pro-inflammatory cytokines and other inflammatory genes. Baseline expression of TNF $\alpha$ , IL-1 $\beta$  and IL-1RA was significantly higher in patients with symptomatic CAD compared to controls (Fig 3A and supplementary Fig 3B), expression of MCP-1, IL-8, IL-6 as well as the expression of HIF-1 $\alpha$ , c-MYC and STAT3 showed no difference.

### Increased intracellular metabolism

We have recently reported that the persistent pro-inflammatory phenotype of monocytes after induction of trained immunity by  $\beta$ -glucan is critically dependent on epigenetic reprogramming and a metabolic shift towards increased aerobic glycolysis<sup>6</sup>. Trained monocytes switch from oxidative phosphorylation to aerobic glycolysis to produce sufficient ATP in an efficient and fast way to maintain the increased inflammatory state. We measured mRNA expression of hexokinase 2 (HK2) and phosphofructokinase 3 (PFKFB3), the two rate-limiting enzymes in the glycolysis pathway, as well as the expression of other important metabolic enzymes in patients with symptomatic CAD and control patients. Patients with symptomatic CAD showed a higher baseline expression of HK2, PFKFB3, pyruvate kinase 1 (PKM1), argininesuccinate lyase (ASL), malate dehydrogenase 1 (MDH1), pyruvate dehydrogenase alpha 1 (PDHA1) and transaldolase 1 (TALDO1) (Fig 3A and Suppl Fig 3B). This is consistent with an upregulation of genes of the glycolytic pathway, but also enzymes in the pentose-phosphate pathway and Krebs cycle (Fig 3B). Expression of HK2 and IL-1b ( $R=0.41$ ,  $p=0.01$ ), PFKFB3 and TNF $\alpha$  ( $R=0.45$ ,  $P=0.006$ ) and PFKFB3 and IL-1 $\beta$  ( $R=0.78$ ,  $p<0.001$ ) are significantly correlated, corroborating an association between upregulated glycolysis and an increased baseline inflammatory state (Suppl Fig 3A).

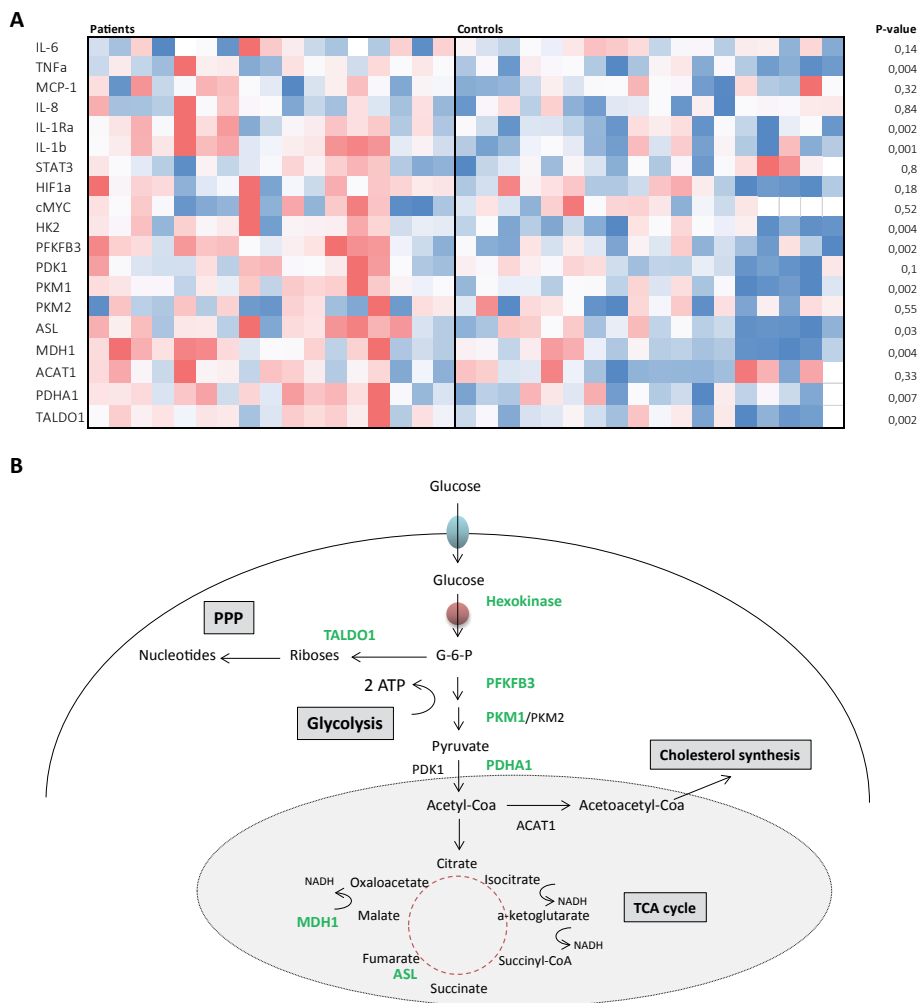
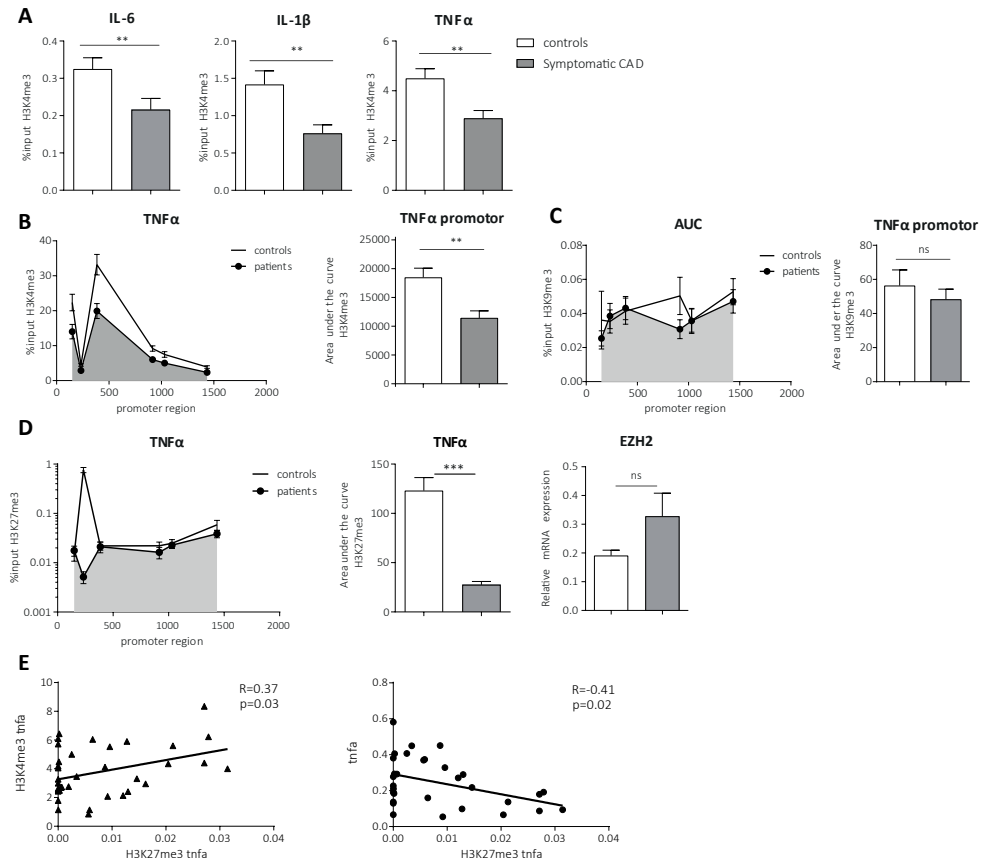


Figure 3. Increased baseline inflammation and glycolysis in patients with symptomatic CAD . (A). Baseline RNA expression of cytokines and metabolic rate-limiting enzymes are significantly increased in patients with symptomatic CAD compared to controls (high expression = red, low expression = blue) (n=17 vs 18) (B). Schematic overview of metabolic pathways and significantly upregulated genes in patients with CAD, highlighted in green.

### Increased cytokine production is associated with lower repressive epigenetic marks in patients with symptomatic CAD

Trained immunity is mediated by epigenetic reprogramming at the level of histone methylation<sup>9</sup>. Previously we showed that increased cytokine production after brief exposure to oxLDL or  $\beta$ -glucan was associated with an enrichment of the activating histone mark H3K4me3, and training was prevented by nonselective inhibitors of methyltransferases<sup>5,10</sup>. Since patients with symptomatic CAD have elevated pro-inflammatory cytokine production

on baseline, as well as increased pro-inflammatory cytokine production upon ex vivo stimulation, we hypothesized that this is due to epigenetic reprogramming of the monocyte population. We assessed H3K4me3 levels on the promoters of IL-6, IL-1 $\beta$  and TNF $\alpha$ , since these were significantly upregulated upon ex vivo stimulation. Surprisingly and in contrast to what we observed in our in vitro experiments with  $\beta$ -glucan and oxLDL-induced training<sup>5</sup>, the promoters of these pro-inflammatory genes had lower H3K4me3 in patients with symptomatic CAD than controls (Fig 4A). Since TNF $\alpha$  showed the most significant increase



**Figure 4.** Patients with symptomatic CAD have a different epigenetic landscape than healthy controls. (A). Chromatin immunoprecipitation of H3K4me3 in monocytes from patients with symptomatic CAD and controls was performed. Levels of H3K4me3 were measured on the promoters of *tnfa*, *il-6* and *il-1b* and were lower in patients compared to controls. (B). The *tnfa* promoter was studied in more detail and showed an overall lower H3K4me3 throughout the promoter region (C). ChIP of repressive mark H3K9me3 showed no significant difference in H3K9me3 on the promoter of *tnfa* in patients compared to controls. (D). ChIP of repressive mark H3K27me3 showed a significantly lower H3K27me3 throughout the *tnfa* promoter in patients compared to controls. The expression of the methyltransferase EZH2 for H3K27me3 showed no difference. (E). The level of H3K27me3 was negatively correlated with the baseline RNA expression of *tnfa*. Levels of H3K4me3 and H3K27me3 are correlated as well ( $n=20$  vs  $20$ , \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ )



in both plasma levels, baseline RNA expression as well as ex vivo production in patients vs. controls, we investigated this cytokine in more detail. Using six primers covering most part of the promoter (suppl. fig 4A), we confirmed consistent downregulation of H3K4me3 throughout the promoter (Fig. 4B).

The overall activation state of the chromatin, however, is dictated not only by the activating mark H3K4me3, but also by alternative activating marks and repressive marks. Two important repressive histone marks are trimethylation of H3K9 and trimethylation of H3K27<sup>11</sup>. H3K27me3 in particular has been associated with control of gene expression upon ex vivo LPS stimulation<sup>12,13</sup>. Using our in vitro model of oxLDL training, we observed long-term lower repressive marks on the promoter regions of pro-inflammatory genes. Both H3K9me3 as well as H3K27me3 in vitro are lower in trained than untrained monocytes for IL-6 and TNF $\alpha$  (suppl. Fig. 4B). Following these observations, we were interested in the repressive marks in our patients. For H3K9me3, no significant differences were observed on the TNF $\alpha$  promoter region (Fig 4C). With regard to H3K27me3, the TNF $\alpha$  promoter showed a lower occupancy throughout the whole promoter ( $p<0.0001$ , Fig 4D). Interestingly, levels of H3K27me3 were correlated to the levels of H3K4me3 on the promoter of TNF $\alpha$  ( $R=0.37$ ,  $p=0.03$ , Fig. 4E) and negatively correlated to the expression of TNF $\alpha$  itself ( $R=-0.41$ ,  $p=0.02$ , fig 4E). Expression of EZH2, the only methyltransferase known for inducing H3K27me3 was not different between patients and controls (Fig. 4D). H3K9me3 and H3K27me3 showed no differences for IL-6 and IL-1 $\beta$  (Suppl. Fig 4C).

## DISCUSSION

The results of this study add additional evidence in favor of the hypothesis that trained innate immunity contributes to the process of atherosclerosis<sup>4</sup>. We demonstrated that circulating monocytes of patients with established atherosclerosis have a pro-inflammatory phenotype, associated with epigenetic remodeling at the level of histone methylation and increased expression of rate limiting enzymes of the glycolysis pathway and the pentose phosphate pathway. Interestingly, this pro-inflammatory phenotype was present only in patients with severe symptomatic coronary atherosclerosis, and not in patients with mild asymptomatic carotid atherosclerosis.

The underlying molecular basis of trained immunity is only partially deciphered and comprises changes in chromatin organization and intracellular metabolism. In human monocytes trained with  $\beta$ -glucan, a shift from oxidative phosphorylation towards aerobic glycolysis through the AKT-mTOR-HIF-1 $\alpha$  pathway was observed, accompanied by changes in histone marks such as H3K4me1, H3K4me3 and H3K27Ac. Both pathways are nonredundant, since pharmacological inhibitors of glycolysis, such as 2-deoxy-D-glucose and inhibitors of the mTOR pathway, such as metformin, as well as nonspecific inhibitors of histone methyltransferases, prevented  $\beta$ -glucan-induced trained immunity<sup>6,10</sup>

Here we show that in patients with symptomatic CAD, monocytes display an increased pro-inflammatory cytokine response upon ex vivo TLR4/2 stimulation. Previously, ex vivo inflammatory responses in patients with and without atherosclerosis were studied mainly in whole blood or peripheral blood mononuclear cells (PBMCs)<sup>14–16</sup>. In addition, baseline expression of cytokines, baseline expression of the two rate-limiting glycolysis genes as well as the expression of several other enzymes in glycolysis, pentose phosphate pathway and Krebs cycle was increased in whole blood. In this study, the pro-inflammatory phenotype was also associated with changes in the epigenetic landscape in the monocytes. First we studied the level of H3K4me3, a long-term activating histone mark. In contrast to our in vitro observations, however, H3K4me3 occupancy on the TNF $\alpha$  promoter was lower in patients with CAD. The accessibility of the chromatin is determined by activating epigenetic marks as well as repressive epigenetic marks. The repressive mark H3K27me3 importantly contributes to the LPS-induced pro-inflammatory cytokine production<sup>12</sup>. Previously, Wierda et al. used immunohistochemistry to show a reduction of global levels of H3K27me3 modification in vessels with advanced plaques, which was not accompanied by alterations in global levels of the corresponding histone methyltransferase EZH2<sup>17</sup>, corresponding to our current findings. Greissel et al. showed in plaque macrophages a significant reduction of repressive

marks H3K9me2 and H3K27me2 with increasing plaque severity<sup>18</sup>. Although measured in plaque macrophages and not in circulating cells, these studies indicate that repressive marks are strongly associated with the severity of atherosclerotic disease. In our study, we also determined H3K9me3 and H3K27me3 on the TNF $\alpha$  promoter. Although H3K9me3 was not significantly different between patients with and without atherosclerosis, H3K27me3 was much lower and correlated to cytokine RNA expression levels. Recently, Schmidt et al. studied the transcriptional regulator network of human inflammatory macrophages by histone marks. They showed that when promoter regions only have H3K4me3 marks, they are in an open chromatin state. When promoter regions are additionally trimethylated on H3K27me3, the promoter region is 'poised' and transcription is prevented<sup>19</sup>. Furthermore, Kruidenier et al. showed that H3K27 demethylation is a critical determinant for LPS induced TNF $\alpha$  production, and higher baseline levels of H3K27me3 can result in lower TNF $\alpha$  production<sup>12</sup>.

Baseline RNA expression analysis showed increased expression of the rate-limiting enzymes of the glycolytic pathway as well as other important rate-limiting metabolic enzymes in patients with severe atherosclerosis. Previously, we have shown that trained immunity depends on a metabolic shift, resulting in increased glycolysis<sup>6,10</sup>. An increase in gene expression of the two rate-limiting glycolytic genes is compatible with this metabolic shift that characterizes trained immunity<sup>10</sup>. The upregulation of other rate-limiting metabolic enzymes involved in the pentose phosphate pathway and Krebs cycle indicates a more general activation of intracellular metabolism, as shown in figure 4B. Recently, Tawakol et al. showed that in plaque macrophages, there is a relationship between PFKFB3 expression and pro-inflammatory activation, via hypoxia-induced upregulation of macrophage glycolysis. The authors showed a linear relationship between macrophage energetics on the one hand and inflammatory activation on the other hand, regulated via PFKFB3 expression<sup>20</sup>. Furthermore, Shirai et al. showed that circulating monocytes and monocyte-derived macrophages from patients with established atherosclerosis have increased inflammation and glycolysis<sup>21</sup>. This study however differs markedly from our study, since these patients already suffered an acute myocardial infarction, which in itself is a well-known trigger of innate immune activation<sup>22</sup>. In our study, all subjects with coronary atherosclerosis were free from previous cardiovascular events. Here we show for the first time that baseline gene expression of glycolysis and intracellular metabolism genes in circulating cells is also increased in event-free patients with established atherosclerosis, which is also associated with changes in the epigenetic landscape.

We propose that the pro-inflammatory phenotype of the circulating monocytes in the patients with severe coronary atherosclerosis reflects a trained immune phenotype, but we can only speculate about the stimulus that has instigated this immunological program. We

have previously shown that several PAMPs and DAMPs can induce trained immunity, including *Bacillus Calmette-Guérin*, *Candida albicans*,  $\beta$ -glucan, and pro-atherogenic stimuli such as modified LDL and Lp(a)<sup>5,8,9,23</sup>. Our patients and controls did not show any differences in total cholesterol levels, LDL cholesterol or Lp(a), but the majority of the patients were being treated with statins. It is probable that before the initiation of statin treatment, LDL was higher in the patients with CAD. Furthermore, the effects of statins on epigenetic modifications are still unknown. HDL cholesterol was lower in the patients, but we do not yet know the effects of HDL cholesterol on trained immunity. Interestingly, the trained immune phenotype of the circulation monocytes was only present in patients with severe symptomatic coronary atherosclerosis, and not in patients with asymptomatic carotid atherosclerosis, which was diagnosed by screening of healthy subjects. A potential explanation for this finding is that trained immunity contributes to the progression of complex atherosclerotic plaques and not to the initial formation of early lesions, which are observed in the majority of subjects. This finding is consistent with previous findings in patients with elevated levels of lipoprotein(a), which can also induce trained immunity in vitro<sup>23</sup>. Interestingly, most studies report that elevated lipoprotein (a) is not associated with early atherosclerotic lesions (e.g. carotid IMT) whereas elevated lipoprotein(a) is consistently associated with cardiovascular events, which mostly result from vulnerable atherosclerotic plaques<sup>24–26</sup>. Obviously, this finding requires confirmation in a larger cohort of patients.

### Limitations

Our study has several potential limitations. First of all, our results only indicate an association between atherosclerosis and trained immunity, and do not provide proof of causality. In theory, effects on the immune system could be caused by the atherosclerosis itself. Intervention studies in animal models of atherosclerosis will be required to provide final proof that trained immunity contributes to the development/progression of atherosclerosis. Secondly, the majority of patients with CAD used statins, in contrast to the control group. However, since statins are known to have potent anti-inflammatory effects<sup>27</sup>, it is very unlikely that they have contributed to the pro-inflammatory monocyte phenotype, although they may have partially masked its full effects. Thirdly, it is important to realize that RNA expression was studied in whole blood using PAXgene tubes, to prevent changes in the RNA expression levels by the isolation of monocytes. However, it is likely that differences in RNA expression that we observed between patients with and without atherosclerosis are relevant for the monocyte fraction: first, we measured expression of various cytokines that are predominantly derived from monocytes, including TNF, IL1b, and IL6. Secondly, there is a strong correlation between the expression of enzymes of the glycolytic pathway (HK2/PFKFB3) and these cytokines, suggesting that this increased glycolysis is relevant for monocytes. Finally, in a previous

study in patients with atherosclerosis, an increased expression of glycolytic enzymes was also observed in isolated monocytes after differentiation into M1 macrophages, compared to monocytes from patients without atherosclerosis<sup>21</sup>.

Finally, with regard to the epigenetic reprogramming, we have only focused on a few epigenetic marks on the promoters of the various cytokines. We studied the ChIPed promoters of genes that were upregulated in protein and RNA expression and searched for association, whereas a more unbiased approach (e.g. genome wide ChIP seq) could reveal new pathways and models for explaining differences.

### **Conclusion**

Here we show that monocytes of patients with symptomatic CAD but not patients with asymptomatic carotid atherosclerosis have a pro-inflammatory phenotype, which is associated with metabolic reprogramming on the level of RNA expression towards increased glycolysis, and with epigenetic remodeling of pro-inflammatory genes. These findings suggest that trained immunity could play a role in the progression of mild and asymptomatic atherosclerotic plaques towards symptomatic disease.

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**Table 1.** Baseline characteristics of included subjects. Data are presented as mean  $\pm$  SD or n (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TChol, total cholesterol; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; Lp(a), lipoprotein(a)

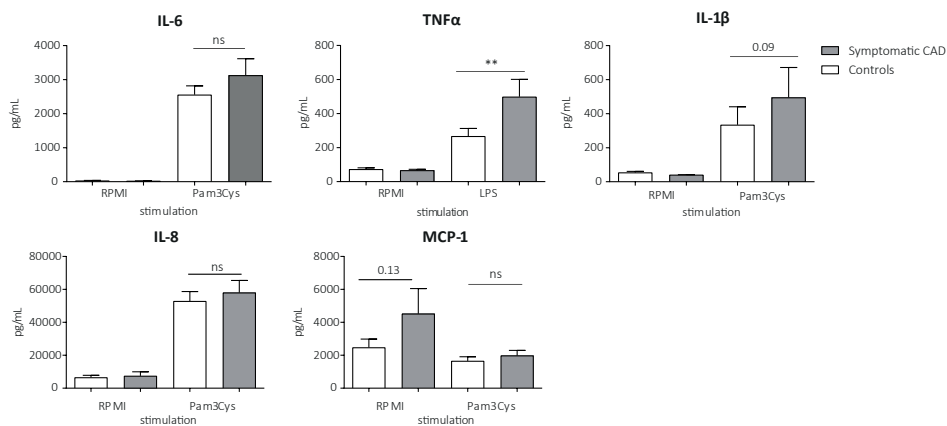
Characteristic	(A) Symptomatic coronary atherosclerosis (n=20)	(B) Controls (n=20)	P value	(C) Asymptomatic carotid atherosclerosis (n=17)	(D) Controls (n=22)	P value
Age, y	52 $\pm$ 11	53 $\pm$ 12	0.78	67 $\pm$ 6	66 $\pm$ 4	0.43
Gender, %male (n)	75 (15)	50 (10)	0.19	29 (5)	45 (10)	0.32
BMI (kg/m <sup>2</sup> )	28.6 $\pm$ 4.1	26.5 $\pm$ 3.5	0.08	29.6 $\pm$ 2.3	31.1 $\pm$ 4.7	0.24
SBP (mmHg)	145 $\pm$ 21	142 $\pm$ 17	0.63	130 $\pm$ 10	130 $\pm$ 14	0.95
DBP (mmHg)	80 $\pm$ 14	83 $\pm$ 11	0.54	79 $\pm$ 6	82 $\pm$ 9	0.22
Family history, %yes (n)	60 (12)	35 (7)	0.21	47 (8)	37 (8)	0.24
Smoking, %active (n)	20 (4)	20 (4)	-	6 (1)	9 (2)	0.72
Smoking (packyears)	18 $\pm$ 12	22 $\pm$ 20	0.47	-	-	-
Statin use, %yes (n)	75 (15)	15 (3)	<b>0.00</b>	0 (0)	0 (0)	1
TChol, mmol/L	5.1 $\pm$ 1.2	4.9 $\pm$ 1.7	0.92	6.2 $\pm$ 0.9	6.0 $\pm$ 1.07	0.55
LDLc, mmol/L	3.0 $\pm$ 1.0	2.9 $\pm$ 1.2	0.93	4.12 $\pm$ 0.8	4.0 $\pm$ 0.9	0.47
HDLc, mmol/L	1.3 $\pm$ 0.3	1.5 $\pm$ 0.6	<b>0.04</b>	1.4 $\pm$ 0.3	1.4 $\pm$ 0.2	0.70
Lp(a), mg/L	281.9 $\pm$ 341.6	266.1 $\pm$ 479.1	0.68	-	-	-
Glucose, mmol/L	6.3 $\pm$ 1.8	5.8 $\pm$ 1.6	0.37	5.4 $\pm$ 0.8	5.3 $\pm$ 0.7	0.71
Creatinin $\mu$ mol/L	86.7 $\pm$ 13.8	80.3 $\pm$ 16.5	0.19	82.2 $\pm$ 12.5	80.1 $\pm$ 17.2	0.67
% Monocytes	12.3 $\pm$ 3.5	13.5 $\pm$ 4.3	0.34	8.6 $\pm$ 1.3	8.9 $\pm$ 2.1	0.65
Total Plaque Score (TPS)	6.3 [4-12]	0	<0.0001	-	-	-
Plaque on carotid ultrasound % (n)	-	-	-	100 (17)	0 (22)	<0.0001



# SUPPLEMENTARY MATERIAL

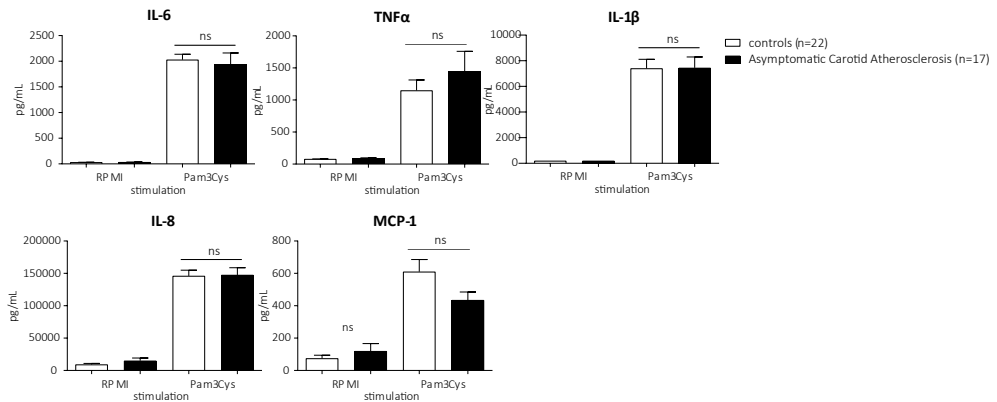
## Supplementary Figure 1

Monocytes from patients with symptomatic CAD have an inflammatory phenotype upon ex vivo stimulation of TLR2 compared to healthy controls. Monocytes from patients and controls were stimulated with TLR2 ligand Pam3Cys for 24h and IL-6, TNF $\alpha$ , IL-1 $\beta$ , IL-8 and MCP-1 were measured in the supernatant after 24h of incubation at 37°C, 5% CO<sub>2</sub> (n=20 vs 20, \* p<0.05, \*\* p<0.01).



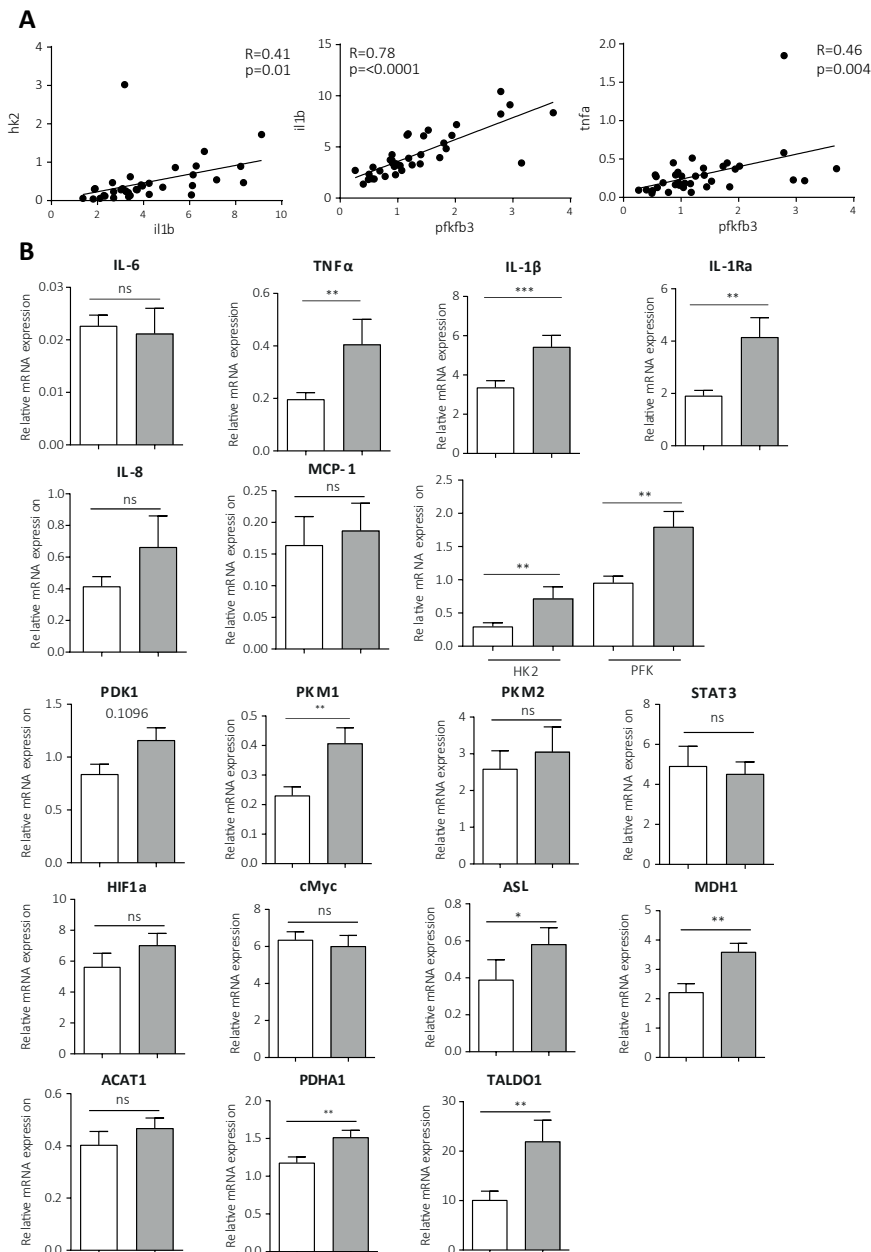
## Supplementary Figure 2

Monocytes from subjects with asymptomatic carotid atherosclerosis do not show an increased inflammatory phenotype upon ex vivo stimulation of TLR2 compared to matched controls. Monocytes from patients and controls were stimulated with TLR2 ligand Pam3Cys for 24h and IL-6, TNF $\alpha$ , IL-1 $\beta$ , IL-8 and MCP-1 were measured in the supernatant after 24h of incubation at 37°C, 5% CO<sub>2</sub> (n=17 vs 22, \* p<0.05, \*\* p<0.01).



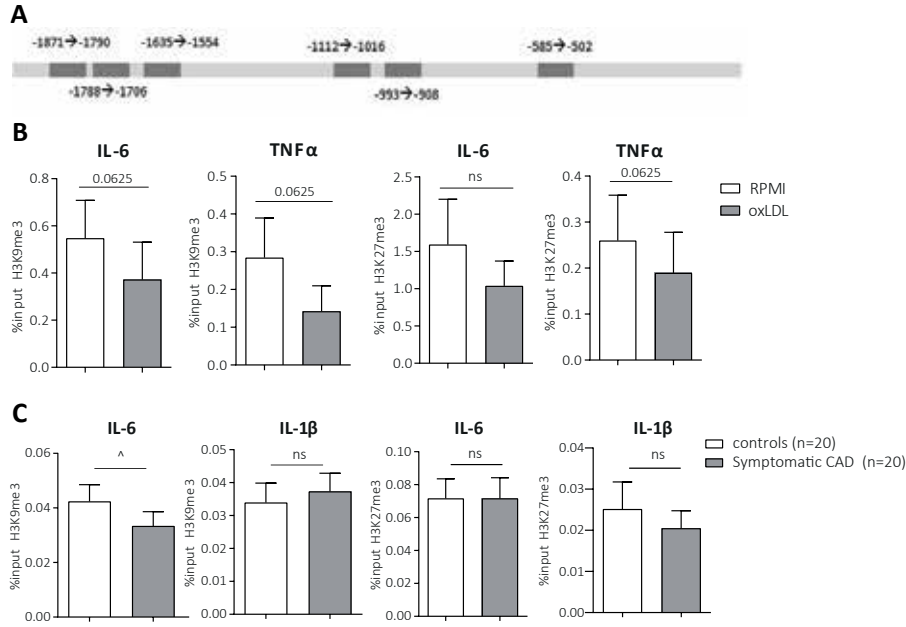
**Supplementary Figure 3**

The pearson correlation coefficient was calculated for baseline RNA expression of cytokines and metabolic rate-limiting enzymes. A. Cytokines TNF $\alpha$  and IL-1 $\beta$  and glycolytic genes hk2 and pfkfb3 are significantly correlated (n=17 vs 18, p<0.05 was considered significant). B. Relative RNA expression of cytokines and metabolic rate-limiting enzymes in symptomatic CAD patients and matched controls (n=17 vs 18)



**Supplementary Figure 4**

A. Schematic overview of the *tnfa* promoter and the designed primers. B. %input H3K9me3 and H3K27me3 of IL-6 and TNF $\alpha$  in vitro trained monocytes (n=4). C. %input H3K9me3 and H3K27me3 of IL-6 and IL-1 $\beta$  in patients and controls (n=20 vs 20).





# PART III

## PATHWAYS OF TRAINED IMMUNITY



# CHAPTER 7

## THE MEVALONATE PATHWAY DRIVES METABOLIC AND EPIGENETIC REPROGRAMMING DURING INDUCTION OF TRAINED IMMUNITY

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## ABSTRACT

Innate immune cells can display adaptive characteristics after infections or vaccinations, a process called trained immunity, which is mediated by interactions between immunological, metabolic and epigenetic pathways. Metabolome and transcriptome analysis of human trained monocytes identified cellular cholesterol synthesis pathway to play an important role in trained immunity. Pharmacological and genetic modulation of the cholesterol synthesis pathway indicated that the metabolite mevalonate is a crucial mediator of trained immunity. Mevalonate induces a trained immunity phenotype in human primary monocytes, accompanied by whole-genome histone modifications of inflammatory pathways. HMG-CoA-reductase inhibitors (statins) that block mevalonate formation prevent trained immunity in terms of cytokine production, glycolysis, and enrichment of H3K4me3 histone marks. Finally, monocytes of patients with hyper IgD syndrome (HIDS) due to mevalonate kinase deficiency demonstrate a trained immunity phenotype at both immunological and epigenetic level, which likely explain their attacks of fever and inflammation. In conclusion, unraveling the role of the mevalonate pathway for the induction of trained immunity contributes to our understanding of the pathophysiology of HIDS, and identified potential novel therapeutic strategies in clinical conditions characterized by excessive induction of trained immunity.

## INTRODUCTION

The immune system is classically divided into the innate and the adaptive immune system. The innate immune system is rapid, relatively non-specific and cannot build immunological memory, whereas the adaptive immune system is slower, but specific and can build memory. However, the recent observation that innate immune cells can exert adaptive characteristics and can build a non-specific memory challenged this paradigm (1). This is supported by a wide variety of recent, but also older studies, in plants, invertebrates and mammals (2, 3). Cells of the innate immune system, such as monocytes and NK cells, are able to build up memory, a process that is dependent on epigenetic changes (4). Once these cells encounter a secondary stimulus, either similar or unrelated to the first insult, their response is altered, which could result both in a stronger or attenuated response. The induction of a non-specific memory resulting in an enhanced function of the innate immune system is termed 'trained (innate) immunity' (1, 2).

Analysis of differentially regulated pathways in trained monocytes and macrophages already revealed an important role for changes in intracellular metabolism. A shift from oxidative phosphorylation to aerobic glycolysis (the Warburg effect), regulated via the mTOR-HIF1 $\alpha$  pathway was identified as a central regulatory mechanism (5). Shifts in cellular metabolism are known to be important drivers of cell phenotype and activation (6). Interestingly, not only glucose metabolism, but also other metabolic pathways play an important role in reprogramming and polarization of cells. Recently, we described that also glutaminolysis and accumulation of fumarate contribute to the trained immunity phenotype, by inhibiting KDM5 demethylases and thus influencing the epigenetic remodeling (7). Here, we seek to unravel how the cholesterol synthesis pathway modulates trained immunity and epigenetic reprogramming; combining transcriptomic and epigenomic studies in both b-glucan and oxLDL trained monocytes. We validated the role of these processes in patients with hyper IgD syndrome (HIDS) that have a defective cholesterol synthesis pathway due to mevalonate kinase deficiency.

## MATERIALS AND METHODS

### Study subjects

Inclusion of healthy controls and HIDS patients was approved by the local institutional review board (CMO region Arnhem-Nijmegen) and conducted according to the principles of the International Conference on Harmonization–Good Clinical Practice guidelines.

### Cells and reagents

Buffy coats from healthy donors were obtained after written informed consent (Sanquin blood bank, Nijmegen, The Netherlands).  $\beta$ -glucan (b-1,3-(D)-glucan) was kindly provided by Professor David Williams (College of Medicine, Johnson City, USA). oxLDL was freshly prepared as previously described (8). Stimuli and pharmacological inhibitors used include lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO; from *E. coli* serotype O55:B5, further purified as described (9)), Pam3Cys (EMC microcollections, Tübingen, Germany), fluvastatin sodium hydrate (Sigma-Aldrich), (R)-Mevalonic acid lithium salt (Sigma-Aldrich), zaragozic acid (SantaCruz Biotechnology), 6-fluoromevalonate (Sigma-Aldrich), 2-deoxyglucose (Sigma-Aldrich), rapamycin (Sigma-Aldrich), methylthioadenosine (Sigma-Aldrich), LXR-inhibitor GSK2033 (1 $\mu$ M, 10 $\mu$ M; Axon Medchem), or PPAR $\delta$ -inhibitor GSK3787 (1 $\mu$ M, 10 $\mu$ M; Tocris).

### PBMC and monocyte isolation

PBMC isolation was performed by dilution of blood in pyrogen-free PBS and differential density centrifugation over Ficoll-Paque (GE healthcare, UK). Cells were washed thrice in PBS. Percoll isolation of monocytes was performed as previously described (10). Briefly, 150–200 $\cdot$ 10<sup>6</sup> PBMCs were layered on top of a hyper-osmotic Percoll solution (48,5% Percoll (Sigma-Aldrich, St Louis, MO, USA), 41,5% sterile H<sub>2</sub>O, 0.16M filter sterilized NaCl) and centrifuged for 15 minutes at 580 g. The interphase layer was isolated and cells were washed with cold PBS. Cells were resuspended in RPMI culture medium (Roswell Park Memorial Institute medium; Invitrogen, CA, USA) supplemented with 50 $\mu$ g/ml gentamicin, 2mM L-glutamine, and 1mM pyruvate and counted. An extra purification step was added by adhering Percoll isolated monocytes to polystyrene flat bottom plates (Corning, NY, USA) for 1 hour at 37°C; subsequently a washing step with warm PBS was performed to yield maximal purity.

### Monocyte training and inhibition experiments

Monocytes were trained as described before (11). Briefly, 100,000 cells were added to flat-bottom 96-well plates. After washing with warm PBS, monocytes were incubated with culture medium only as a negative control or 5 $\mu$ g/ml of  $\beta$ -glucan, 10  $\mu$ g/ml oxLDL or 100–1000  $\mu$ M (R)-mevalonic acid for 24h (in 10% pooled human serum). Cells were washed once with 200

μl of warm PBS and incubated for 5 days in culture medium with 10% pooled human serum and medium was changed once. Cells were restimulated with either 200μl RPMI, LPS 10 ng/ml, or Pam3Cys 10 μg/ml. After 24h supernatants were collected and stored at -20°C. In some experiments, cells were pre-incubated (before β-glucan or oxLDL training) for 1 hour with 20 μM fluvastatin sodium hydrate, 1-5 μM zaragozic acid, or 200 μM 6-fluoromevalonate. Concentrations were chosen as being highest non-toxic concentrations, based on pilot-experiments (not shown).

### Mouse experiments

Blood mononuclear cells from wild type mice were isolated using the density gradient cell separation medium histopaque 1083 (Sigma). Cells were incubated with β-glucan (5μg/ml) or mevalonate (500 μM) for 24h in the presence of 20ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech). Cells were then washed with PBS and were cultured until day 6 in 10% fetal bovine serum. Afterwards, they were stimulated with LPS (10ng/ml) for 24h in RPMI without fetal bovine serum. Levels of TNF and IL-6 were measured in cell culture supernatants.

The effect of fluvastatin (20μM) and potent inhibitors for LXR (1μM, 10μM; GSK2033, Axon Medchem) or PPARδ (1μM, 10μM; GSK3787, Tocris) on β-glucan-induced training was examined. Compounds were added during the first 24h. Treatment of cells with dimethyl sulfoxide (DMSO; Sigma) served as control in the case of LXR and PPARδ antagonism.

### Cytokine and lactate measurements

Cytokine production was determined in supernatants using commercial ELISA kits for human IL-1β, TNFα (R&D systems, MN, USA), human IL-6 (Sanquin, Amsterdam, The Netherlands), and mouse TNFα and IL-6 (Meso Scale Discovery) following the instructions of the manufacturer. Lactate concentration was measured using a Lactate Fluorometric Assay Kit (Biovision, CA, USA).

### Foam cell formation

To induce foam cell formation at day 6, trained macrophages were incubated with RPMI alone or RPMI containing oxidized LDL at 50 μg/mL for 24h. Incubation with this concentration of oxLDL alone caused no secretion of IL-6, TNFα or MCP-1, suggesting absence of endotoxin in the prepared LDL fractions. Accumulation of oxLDL was visualized by Oil-Red-O staining as described before (12).

### RNA sequencing and chromatin immunoprecipitation

10x10<sup>6</sup> monocytes were trained in vitro with β-glucan, oxLDL or R-mevalonate in 10cm

petridishes in 10ml medium volumes. After resting for 5 days in culture medium, the cells were harvested for RNA extraction or chromatin immunoprecipitation.

### RNA extraction and cDNA synthesis

Monocytes isolated from HIDS patients and controls, and in vitro derived naive and mevalonate exposed macrophages, were lysed in Trizol reagent and stored at -80°C (Life Technologies). Total RNA was extracted from cells using the Qiagen RNeasy RNA extraction kit (Qiagen, Netherlands), using on-column DNaseI treatment. Ribosomal RNA was removed using the riboZero rRNA removal kit (Illumina). RNA was then fragmented into 200bp fragments by incubation for 4 minutes at 95°C in fragmentation buffer (200 mM Tris-acetate, 500 mM Potassium Acetate, 150 mM Magnesium Acetate, pH 8.2). First strand cDNA synthesis was performed using SuperScript III (Life Technologies), followed by synthesis of the second cDNA strand. Library preparation was performed using the KAPA hyperprep kit (KAPA Biosystems), using the USER enzyme to degrade the second cDNA strand. Quality of cDNA and the efficiency of ribosomal RNA removal was confirmed using quantitative RT-PCR using the IQ Sybr Supermix, with primers for GAPDH, 18S and 28S rRNA.

### Chromatin immunoprecipitation (ChIP)

Purified cells were fixed with 1% formaldehyde (Sigma-Aldrich) at a concentration of approximately 15 million cells/ml. Fixed cell preparations were sonicated using a Diagenode Bioruptor Pico sonicator using 5 cycles of 30 seconds on, 30 seconds off. One µg of chromatin (approx. 0.5-1 million cells) was incubated with dilution buffer, 12 µl protease inhibitor cocktail and 1µg of H3K27ac antibody or H3K4me3 (Diagenode) to a final volume of 300 µl, and incubated overnight at 4 °C with rotation. Protein A/G magnetic beads were washed in dilution buffer with 0.15% SDS and 0.1% BSA, added to the chromatin/antibody mix and rotated for 60 minutes at 4°C. Beads were washed with 400µl buffer for 5 minutes at 4 °C with five rounds of washes. After washing chromatin was eluted using 200 µl elution buffer for 20 minutes. Supernatant was collected, 8 µl 5M NaCl, 3µl proteinase K were added and samples were incubated for 4 hours at 65°C. Finally samples were purified using Qiagen Qiaquick MinElute PCR purification Kit and eluted in 20 µl elution buffer.

For qPCR analysis, samples were analyzed using a comparative Ct method in which myoglobin was used as a negative control and H2B as a positive control according to the manufacturer's instructions. The following primers were used for this analysis: myoglobulin forward AGCATGGTGCCACTGTGCT; myoglobulin reverse GGCTTAATCTCTGCCTCATGAT; H2B forward TGTACTTGGTGACGGCCTTA; H2B reverse CATTACAACAAGCGCTCGAC; TNFA forward GTGCTTGTTCTCAGCCTCT; TNFA reverse ATCACTCCAAAGTGCAGCAG; IL-6 forward AGGGAGAGCCAGAACACAGA; IL-6 reverse GAGTTTCCTCTGACTCCATCG.

#### Library preparation for sequencing

Illumina library preparation was done using the Kapa Hyper Prep Kit (Kapa Biosystems). For end repair and A-tailing double stranded DNA was incubated with end repair and A-tailing buffer and enzyme and incubated first for 30 minutes at 20 °C and then for 30 minutes at 65°C. Subsequently adapters were ligated by adding 30µl ligation buffer, 10 Kapa I DNA ligase, 5 µl diluted adaptor in a total volume of 110µl and incubated for 15 minutes at 15 °C. Post-ligation cleanup was performed using Agencourt AMPure XP reagent and products were eluted in 20 µl elution buffer. Libraries were amplified by adding 25 µl 2x KAPA HiFi Hotstart ReadyMix and 5µl 10x Library Amplification Primer Mix and PCR, 10 cycles. Samples were purified using the QIAquick MinElute PCR purification kit and 300bp fragments selected using E-gel. Correct size selection was confirmed by BioAnalyzer analysis. Sequencing was performed using the Illumina NextSeq500 machine and generated 43bp paired end reads. Samples for RNA-seq were treated to the above protocol exactly, except for a single additional step: After post-ligation cleanup, and before library amplification, samples were incubated with 3 µL USER enzyme for 15 minutes at 37°C to digest the second cDNA strand.

#### RNA-seq data analysis

To infer gene expression levels, RNA-seq reads were aligned to the Ensembl v68 human transcriptome using Bowtie 1 (13). Quantification of gene expression was performed using MMSEQ (<https://github.com/eturro/mmseq>) (14) Haplotype and isoform specific expression estimation using multi-mapping RNA-seq reads. Differential expression was determined using DEseq (<http://bioconductor.org/packages/release/bioc/html/DESeq.html>) (15) Differentially expressed genes were determined using pair-wise comparison between treatments, with a fold change >1.5, adjusted p value of <0.1, and RPKM >1.

#### ChIP-seq data analysis

Sequencing reads were aligned to human genome assembly hg19 (NCBI version 37) using bwa (16). Duplicate and low quality reads were removed after alignment using Samtools and Bamtools (17, 18). For peak calling the BAM files were first filtered to remove the reads with mapping quality less than 15, followed by fragment size modeling (<http://code.google.com/p/phantompeakqual-tools/>). MACS2 (<http://github.com/taoliu/MACS/>) was used to call the peaks (19). H3K27ac peaks were called using the default (narrow) setting. Reads/peak tables were merged using Bedtools (20). Data were normalized using the R package DESeq1 and then pair-wise comparisons were performed (fold change 1.5, adjusted p-value <0.1 and reads/peak ≥10 in at least in any condition) to determine the differential peaks.

## **Statistics**

In-vitro monocyte experiments were performed at least 6 times and analyzed using a Wilcoxon signed-rank test or one-way ANOVA, where applicable. A P-value below 0.05 was considered statistically significant. All data were analyzed using Graphpad prism 5.0 (La Jolla, CA, USA). \*  $p < 0.05$ , \*\*  $p < 0.01$ . Data are shown as means  $\pm$  SEM.

## RESULTS

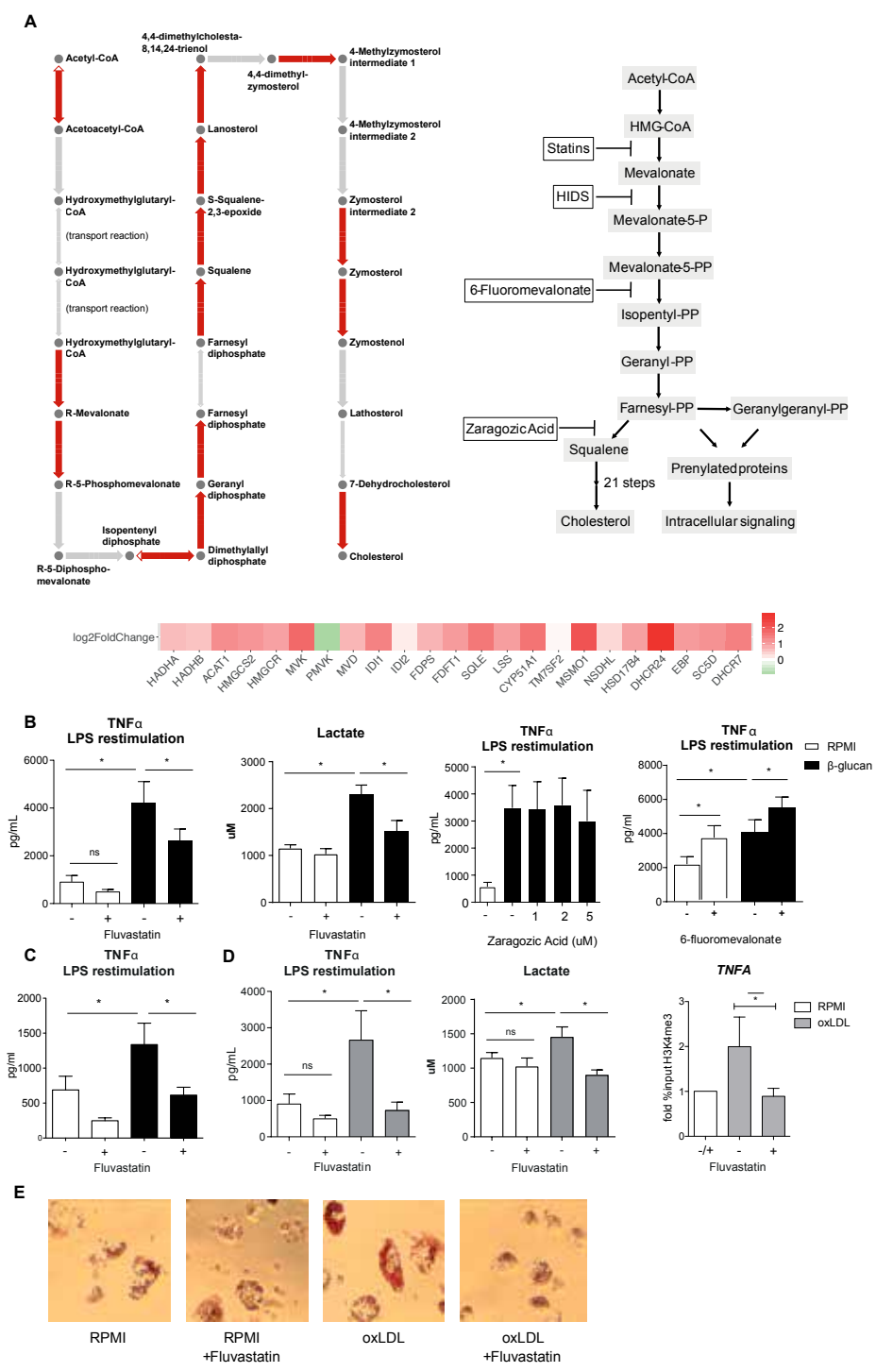
### **Activation of the cholesterol synthesis pathway, but not the synthesis of cholesterol itself is essential for induction of trained immunity**

B-glucan can induce a long-term reprogramming in monocytes and macrophages, resulting in enhanced function (21). Integrating the transcriptome and metabolome of b-glucan-trained macrophages showed an upregulation of several major metabolic pathways, such as glucose metabolism, glutaminolysis, and the cholesterol synthesis pathway (5, 7). The expression of several key enzymes in the cholesterol synthesis pathway was strongly increased by b-glucan training (Fig 1A), leading to the hypothesis that the cholesterol synthesis pathway contributes to trained immunity.

Pharmacological inhibition of the rate-limiting enzyme HMG-CoA-reductase with fluvastatin prevented the induction of trained immunity by b-glucan, illustrated by decreased cytokine production upon re-stimulation. Moreover, lactate production was also decreased, indicating reduced glycolysis, which is an important component of the trained immunity phenotype (Fig 1B) (7). Additionally, when mouse mononuclear cells were trained with b-glucan, the induction of cytokine production upon LPS restimulation could also be inhibited with fluvastatin (Fig 1C). To further unravel which part of the cholesterol pathway is essential for this effect, several inhibitors of enzymes downstream of HMG-CoA-reductase were assessed for their capacity to modulate trained immunity (Fig 1A,B). To study whether cholesterol synthesis itself is necessary for training, cells were preincubated with zaragozic acid A, which inhibits squalene synthase. Preincubation of monocytes with zaragozic acid, however, did not inhibit the induction of pro-inflammatory cytokine production (Fig 1B), indicating that cholesterol itself is not essential for trained immunity. Secondly, 6-fluoromevalonate was used to inhibit mevalonate pyrophosphate (PP) decarboxylase. Mevalonate PP decarboxylase catalyzes the formation of isopentenyl-PP from mevalonate-5-PP. Pre-incubation of monocytes with 6-fluoromevalonate augmented the induction of pro-inflammatory cytokine production by  $\beta$ -glucan (Fig 1B), indicating that the accumulation of a molecule upstream of 6-fluoromevalonate plays a role in the induction of cytokine production, and excluding a role for protein prenylation.

We have previously reported that brief exposure to oxLDL also induced trained immunity (22). Pre-incubation of cells with fluvastatin also prevented oxLDL-induced trained immunity, both in terms of cytokine production upon restimulation and decreased lactate production and glycolysis. Incubation with fluvastatin prevented epigenetic reprogramming of both oxLDL and b-glucan training (Fig 1D, (7)), and also prevented increased foam cell formation,





*Figure 1 - The cholesterol synthesis pathway in trained immunity. (A) Transcriptional expression of macrophages five days after  $\beta$ -glucan exposure. Red arrow = significant upregulation. Overview of the cholesterol synthesis pathway and the used inhibitors. (B) Monocytes were trained with  $\beta$ -glucan +/- inhibitors of the cholesterol synthesis pathway. Five days after  $\beta$ -glucan exposure, lactate concentrations were measured in the supernatants and macrophages were restimulated for 24h with LPS and TNF $\alpha$  concentrations were measured in the supernatant. (N=6, mean  $\pm$  SEM). (C) Mouse blood mononuclear cells were trained with  $\beta$ -glucan +/- fluvastatin on day 1. At day 6 cells were stimulated for 24h and cytokines were determined in the supernatants. (N=6, mean  $\pm$  SEM). (D) Monocytes were trained with oxLDL +/- fluvastatin for 24h. Five days later, epigenetic (H3K4me3) changes at the TNFA promoter and lactate concentrations in the supernatant were measured. Macrophages were also restimulated for 24h with LPS to determine TNF $\alpha$  concentrations in the supernatant. (N=6, mean  $\pm$  SEM). (E) Monocytes were trained with oxLDL +/- fluvastatin for 24h. Five days later foam cell formation was induced by exposure to high concentration of oxLDL. After 24h cells were stained with Oil-Red-O.*

which was induced by training with oxLDL (Fig 1E). OxLDL- and  $\beta$ -glucan-induced training was associated with enrichment of H3K4me3 on the promoter TNFA, which was prevented by fluvastatin (Fig 1D, (7)).

### Mevalonate induces trained immunity by epigenetic reprogramming

As the induction of trained immunity is inhibited by fluvastatin and increased with 6-fluoromevalonate, we came to the hypothesis that mevalonate is the metabolite from the cholesterol synthesis pathway that induces a trained immunity phenotype. Indeed, addition of exogenous mevalonate recaptured the  $\beta$ -glucan- and oxLDL-induced trained phenotype in the presence of fluvastatin (Fig 2A). We further investigated the induction of training by mevalonate and found that incubation of monocytes with mevalonate on day 0 increased cytokine production upon restimulation 6 days later (Fig 2B). Again, this could be reproduced in mouse monocytes (Fig 2C). Furthermore, pre-incubation with 6-fluoromevalonate potentiated the effect of mevalonate (Fig 2D). Interestingly, also lactate production was increased in mevalonate-trained macrophages (Fig 2E), which suggests activation of the glycolytic pathway similar to  $\beta$ -glucan-trained macrophages (5). Indeed, inhibition of glycolysis with 2-deoxyglucose and inhibition of mTOR with rapamycin prevented mevalonate-induced trained immunity (Fig 2F). This shows a crucial role for glucose metabolism and mTOR signaling in mevalonate-induced trained immunity. In addition, to determine the role of histone methylation in the induction of trained immunity by mevalonate, we added methylthioadenosine (MTA, a non-selective methyltransferase inhibitor) to the in-vitro system during the first 24h. Addition of MTA prevented mevalonate-induced trained immunity in terms of increased cytokine production (Fig 2G). Indeed, mevalonate induced an enrichment of H3K4me3 on the promoters of IL6 and TNFA (Fig 2H), similar to the training by  $\beta$ -glucan and oxLDL (21, 22).

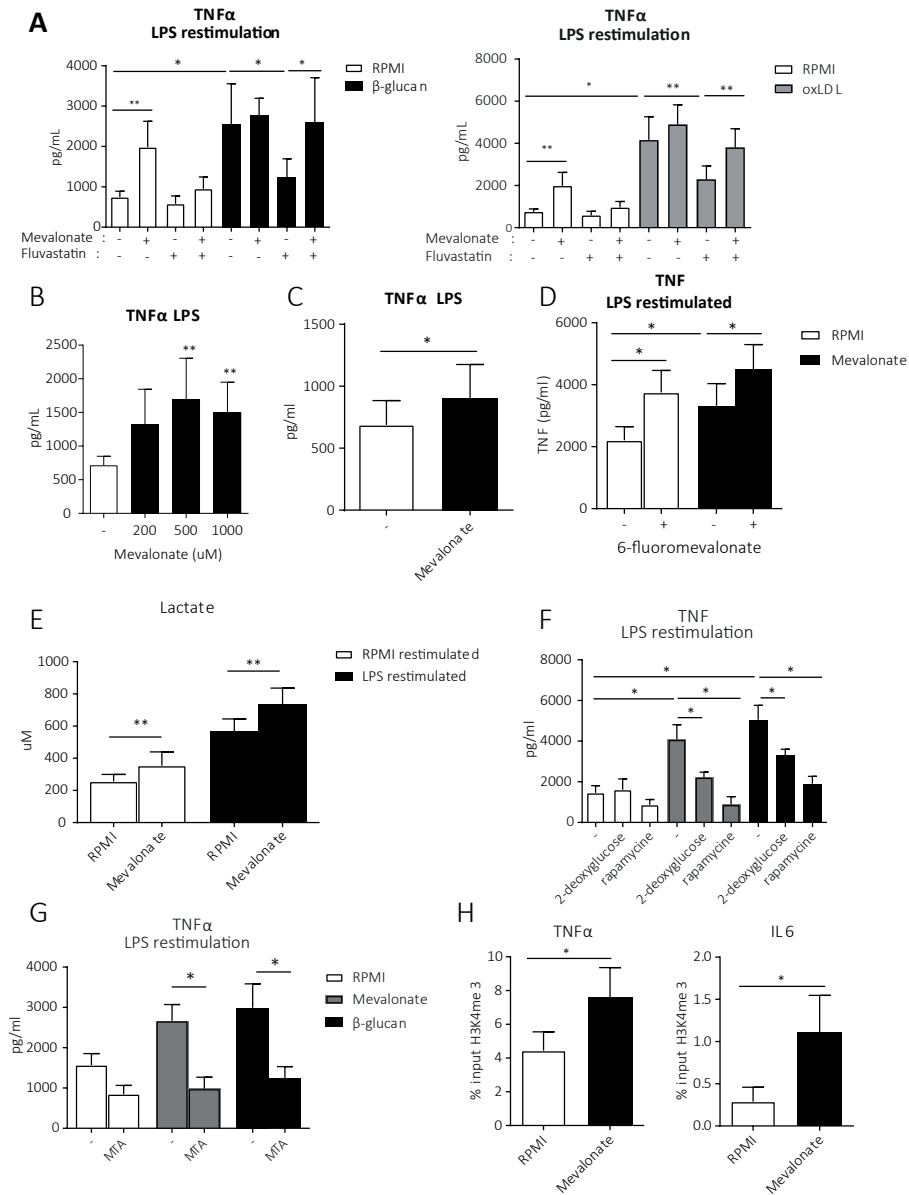


Figure 2 - Mevalonate induces trained immunity. (A) Adding mevalonate during the first 24h can rescue the inhibition of oxLDL/β-glucan-induced training by fluvastatin. (N=6, mean ± SEM). (B) Human and (C) mouse monocytes were trained with mevalonate for 24h and restimulated with LPS at day 6. TNFα concentration were measured in the supernatants. (N=6, mean ± SEM). (D) When 6-fluoromevalonate was added during the first 24h, training with mevalonate could be enhanced. (N=6, mean ± SEM). (E) Lactate production of RPMI or LPS re-stimulated mevalonate-trained macrophages at day 6. (N=10, mean ± SEM). (F) When 2-deoxyglucose (2-DG) or rapamycin were added during the first 24h, mevalonate and β-glucan induced training could be inhibited. (N=6, mean ± SEM). (G) Adding a methyltransferase inhibitor (MTA) inhibits the induction of trained immunity. (N=6, mean ± SEM). (H) H3K4me3 at promoters of TNFA and IL6 was assessed at day 5 of mevalonate-trained macrophages. (N=6, mean ± SEM).

To further elucidate the mechanism through which mevalonate induces trained immunity, we performed RNA-sequencing and a whole-genome assessment of the histone mark H3K27ac by ChIP-sequencing in mevalonate-trained macrophages. Monocytes were trained as described above, and after 5 days of rest (before restimulation), monocyte-derived-macrophages were harvested for ChIP- and RNA-sequencing (Fig 3A). Similar to fumarate-trained macrophages no consistent differences were found in transcription profiles at this time point (7). This is consistent with previous observation for  $\beta$ -glucan trained macrophages that the major differences in gene expression are present during the first 24h after  $\beta$ -glucan stimulation, whereas at later time points transcriptional differences are less pronounced (23). Whole genome epigenetic analysis of mevalonate-trained macrophages, by chromatin immunoprecipitation of histone 3 lysine 27 acetylation (H3K27ac) did reveal noticeable differences between trained and non-trained macrophages. In total 223 defined peaks showed an upregulation of H3K27ac and 554 peaks showed a downregulation in mevalonate-trained macrophages (Fig 3B-D, Table S1). When these peaks were compared with  $\beta$ -glucan-trained macrophages, of the 223-upregulated peaks only few were also found in  $\beta$ -glucan-trained macrophages. However, of the 554 downregulated peaks 397 were also found in the  $\beta$ -glucan-trained macrophages. Of these peaks 45% also showed a downregulation of at least 2-fold, showing a comparable signature in mevalonate and  $\beta$ -glucan-induced trained immunity (Table S1). Pathway analysis of these differentially regulated peaks in mevalonate-trained monocytes, can be found in Fig 3E and shows the modulation of several important inflammatory pathways.

### **Monocytes of HIDS patients with mevalonate kinase deficiency have a trained immunity phenotype with higher cytokine production**

Mevalonate kinase deficiency in humans leads to Hyper-IgD syndrome (HIDS), an autoinflammatory disorder characterized by an accumulation of mevalonate (24, 25). HIDS patients experience periodic attacks of sterile inflammation with symptoms such as fever, skin lesions, lymphadenopathy, and arthralgia (26). Interestingly, white blood cells of HIDS patients were described to produce more cytokines, unstimulated as well as stimulated, indicating a pre-activated state (27). We hypothesized that this is due to a trained immunity phenotype of HIDS monocytes, likely induced by the accumulation of mevalonate. We investigated the cells of 3 adult HIDS patients (2 male, 1 female), all three with V377I mutations. In line with our hypothesis, purified monocytes from these patients produced significantly more TNF $\alpha$ , IL-6 and IL-1 $\beta$  upon stimulation with several ligands compared to healthy controls (Fig 4A). RNAseq analysis revealed that HIDS monocytes incubated in medium for 24h in the absence of exogenous stimulation display an overexpression of multiple genes (Fig 4B, Table S2), although with substantial interdonor variation (Fig S1). Pathway analysis of these expression data is

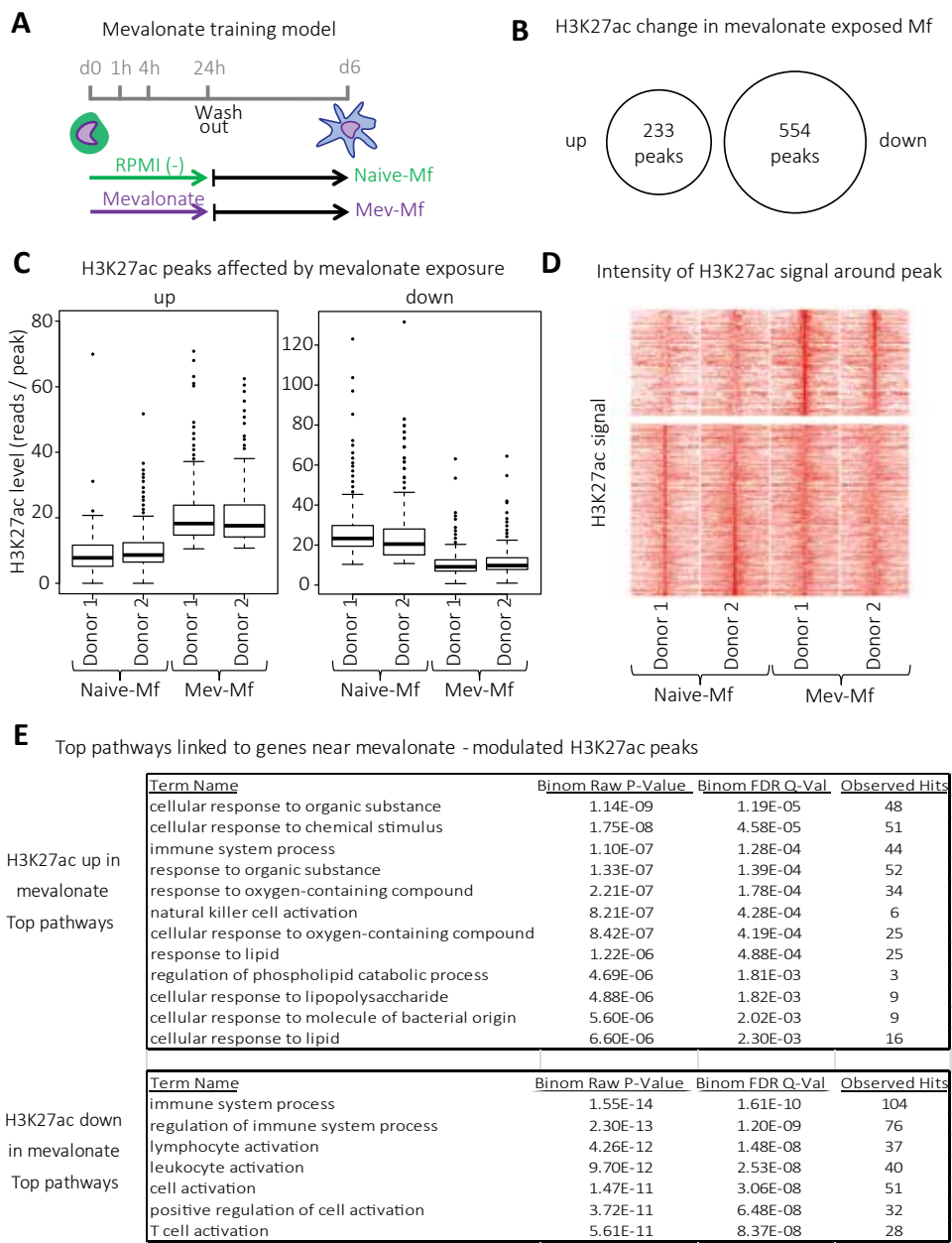


Figure 3 - Epigenetic changes induced by mevalonate. (A) Experimental set-up for mevalonate-induced trained macrophages. (B) Summary of H3K27ac peaks that show higher or lower deposition in mevalonate-trained cells relative to control macrophages. (C) Boxplots of H3K27ac peaks. (D) Heatmap of H3K27ac intensity at peaks that show increased and decreased H3K27ac in mevalonate-trained macrophages. Data is plotted over an area of +/- 12kb from peak center. (E) Pathway analysis of H3K27ac differentially regulated genes. (N=2)

displayed in Fig 4C and shows upregulated inflammatory pathways, such as NFkB signalling, supporting the hypothesis of a hyperinflammatory state. We performed whole-genome assessment of the histone mark H3K27ac by ChIP-sequencing in non-stimulated monocytes from the HIDS patients and controls. Due to variation between the three HIDS patients, only 147 upregulated and 63 downregulated peaks were found to be different comparing HIDS patient to healthy controls (Table S3). The overlap between the in-vitro mevalonate trained macrophages and HIDS circulating monocytes in terms of H3K27ac peaks was limited, due to differences in the cell types analysed (Fig S2). When specifically comparing peak-related genes between HIDS and mevalonate-activated samples one upregulated gene and 22 downregulated genes were found (Table S3), but these could not be allocated to certain cellular pathways, because of the low number. When specifically assessing the expression data of HIDS patients for trained immunity-related genes an interesting comparison can be made. Expression profiles of pathways known to be important for induction of trained immunity such as glycolysis, mTOR pathway, and (innate) cytokines were assessed (4, 5, 21), comparing HIDS patients and healthy controls. This shows a clear increased induction of glycolysis and mTOR pathway in HIDS patients when their monocytes are stimulated with LPS (Fig 4D). Moreover, transcription of cytokines was evidently increased in monocytes of HIDS patients after 24h in RPMI medium, as was shown by increased production upon stimulation before (Fig 4A,D).

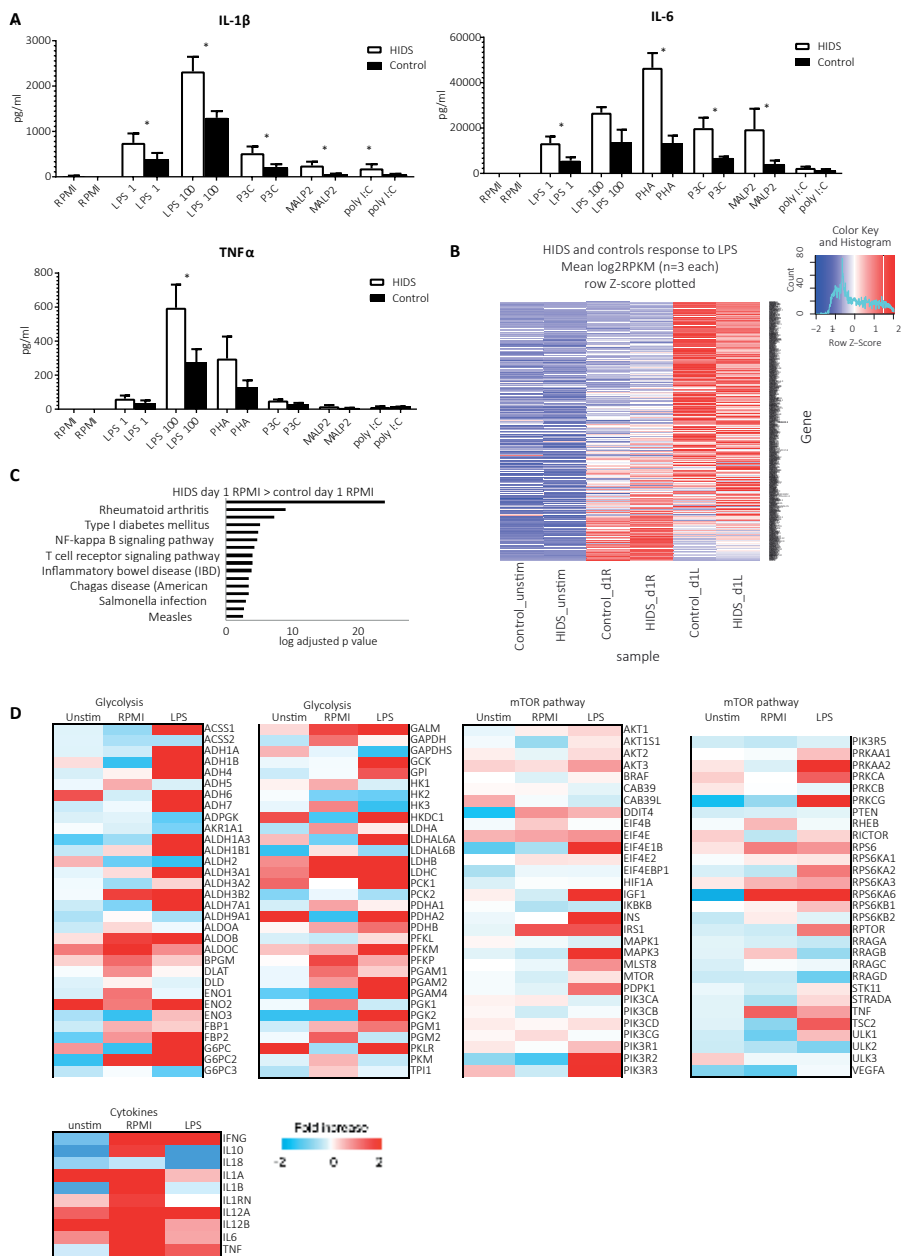


Figure 4 - HIDS patients display a trained-immunity like phenotype. (A) Monocytes from HIDS patients and matched healthy volunteers were exposed ex vivo to several ligands for 24h. Cytokine were measured in the supernatants. (N=4, mean  $\pm$  SEM). (B) Heatmap showing expression data of monocytes of HIDS patients versus healthy volunteers either unstimulated, or 24h stimulated with RPMI or LPS. (N=3 vs N=3). (C) Top pathways associated with genes that show higher expression in HIDS patients exposed to 24h RPMI compared to controls. (N=3 vs N=3). (D) Monocytes of 3 HIDS patients and 3 healthy volunteers were exposed to RPMI/LPS for 24h or left unstimulated (unstim). Fold increases of HIDS/controls were calculated for genes in the glycolysis pathway, mTOR pathway or cytokines. (N=3 vs N=3).

## DISCUSSION

Trained immunity is associated with an upregulation of several cellular metabolic pathways, including glycolysis and glutamine metabolism. In this paper, we explored the role of the mevalonate-cholesterol pathway in the induction of trained immunity. We show that the cholesterol synthesis pathway is also essential for inducing trained immunity. Inhibition of the cholesterol synthesis pathway by statins down-regulated the increased cytokine production and epigenetic reprogramming induced by  $\beta$ -glucan as well as oxLDL. We also show that not the synthesis of cholesterol itself is essential, but the accumulation of mevalonate.

Transcriptome and epigenome analysis showed that incubation of healthy monocytes with mevalonate alone results in trained macrophages that resemble the  $\beta$ -glucan-induced trained immunity phenotype. This indicates that next to fumarate, mevalonate is an essential metabolite in the metabolo-epigenetics of trained immunity. RNA sequencing analysis showed that monocytes from patients with HIDS due to mevalonate kinase deficiency exhibit a trained immunity phenotype.

The cholesterol synthesis pathway has been shown to affect epigenetic remodeling via induction of LXR $\alpha$ , which is induced by intermediates downstream in the cholesterol synthesis pathway (28, 29). Furthermore, downregulation of geranylgeranylation downregulates HDAC1 activity and FPP and GGPP have been shown to inhibit the lysine specific demethylase 1 (30, 31). However surprisingly, in the present study we show that intermediates downstream in the pathway are not essential for inducing trained immunity, while metabolites in the proximal part of the pathway, especially mevalonate, play an important role. We, thus, show that it is unlikely that isoprenylation plays a major role for the induction of trained immunity.

HIDS is one of the prominent hereditary autoinflammatory ('periodic fever') syndromes. HIDS patients carry mutations in mevalonate kinase that are responsible for the clinical picture of febrile attacks, skin lesions, arthritis and lymphadenopathy. The mutation in mevalonate kinase leads to accumulation of mevalonate and results in elevated excretion of mevalonic acid during attacks (25). In the treatment of HIDS inhibition of IL-1 with anakinra has become one of the cornerstones of the therapy (32), but HIDS is considered more and more a 'multi-cytokine disease', as also inhibition of IL-6 with tocilizumab and even anti-TNF treatment with etanercept have shown promising results in specific cases (33-36). We thus hypothesized that induction of a trained immunity phenotype, characterized by exaggerated cytokine production by monocytes due to mevalonate accumulation, could play a role in the pathophysiology of HIDS. This hypothesis was validated by both demonstration of exaggerated cytokine production in HIDS monocytes, accompanied by transcriptional and



epigenetic changes. Interestingly, statin treatment showed a reduced excretion of mevalonic acid in HIDS patients, and a decreased number of febrile days, supporting the concept that lowering mevalonate levels is beneficial for disease activity (37).

Kastner et al. have shown that isoprenylation is an important mechanism that is deficient in HIDS patients and causative to the disease symptoms. The absence of protein geranylgeranylation results in compromised PI(3)K activity, which allows an unchecked TLR-induced inflammatory response with constitutive activation of the pyrin inflammasome. Moreover, due to prenylation defects the GTPase RhoA is inactivated, which also results in pyrin inflammasome activation (38, 39). Interestingly, it was also shown that isoprenylation is not defective in HIDS patients due to increased flux through the isoprenoid/cholesterol biosynthesis pathway by elevating intracellular mevalonate levels (40). However, we now show that next to this mechanism based on defective prenylation, also the accumulation of mevalonate itself might play a role in developing the hyperinflammatory state observed in HIDS.

A crucial aspect relates to the mechanisms through which mevalonate induces and modulates trained immunity. In T-cells, activation induces a shift from oxidative phosphorylation to aerobic glycolysis, similar to trained monocytes. In these cells it was shown that the increased glycolysis also fuels mevalonate metabolism (41). Glucose is increasingly taken up and converted into pyruvate. Subsequently, via a truncated TCA cycle, pyruvate is converted into acetyl-CoA and citrate, which is exported to the cytosol. There, the citrate is converted back into acetyl-CoA by ATP citrate lyase (42) and enters either the mevalonate pathway or fatty acid biosynthesis (41). Previously we have shown an increase in intracellular citrate in  $\beta$ -glucan trained cells, which might provide evidence of a similar mechanism in monocytes as well (7). An interesting observation of our study is the involvement of glycolysis and mTOR for the induction of trained immunity by mevalonate. Mevalonate-induced training can be counteracted by inhibiting glycolysis and mTOR signaling. The mechanism through which mevalonate induces this effect remains to be identified, but mevalonate could exert this effect either intra- or extracellularly. One possibility is activation of an LXR receptor by mevalonate, followed by transcriptional and epigenetic changes leading to shifts of glucose metabolism as well. Another scenario may involve release of mevalonate from the cells during induction of trained immunity, which can act in turn in an autocrine and paracrine manner and induce the observed effects on trained immunity. This mechanism is supported by the observation that mevalonate is able to signal via the IGF1 receptor (43).

Clinically, the discovery of a role for mevalonate for induction of trained immunity, and of a hyper-inflammatory phenotype in specific diseases, has important consequences. Inhibition of the cholesterol synthesis pathway in inflammatory conditions is thus potentially very relevant. Earlier studies have already shown that statins can improve survival from sepsis in mice (44) and also human studies demonstrate that statin use may be associated with better survival in sepsis (45). Also the development of rheumatoid arthritis is thought to be reduced by statin treatment (46). Inhibition of the cholesterol synthesis pathway could therefore be an effective therapy in other hyper-inflammatory disorders in which trained immunity plays a role (1). However, to fully implement inhibition of the mevalonate pathway as a therapeutic option the exact role of trained immunity and mevalonate accumulation in these conditions should be first elucidated further.

In conclusion, the cholesterol synthesis pathway, and in particular the intermediate mevalonate, is essential for inducing trained innate immunity. Incubation of monocytes with mevalonate induces a trained immunity phenotype, which covers a part of the  $\beta$ -glucan trained immunity phenotype, similar to fumarate. Patients with mevalonate accumulation acquire a trained immunity phenotype, by showing increased expression of cytokines and genes in the glycolysis pathway. Furthermore, inhibition of the cholesterol synthesis pathway by statins reduces the trained immunity phenotype. Statins could therefore be used as a treatment option for diseases, which are mediated via trained immunity, until more specific drugs for inhibiting trained immunity are available.

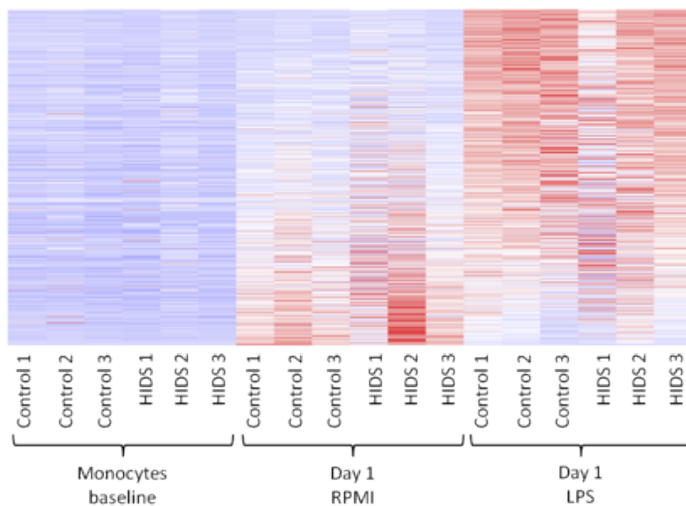
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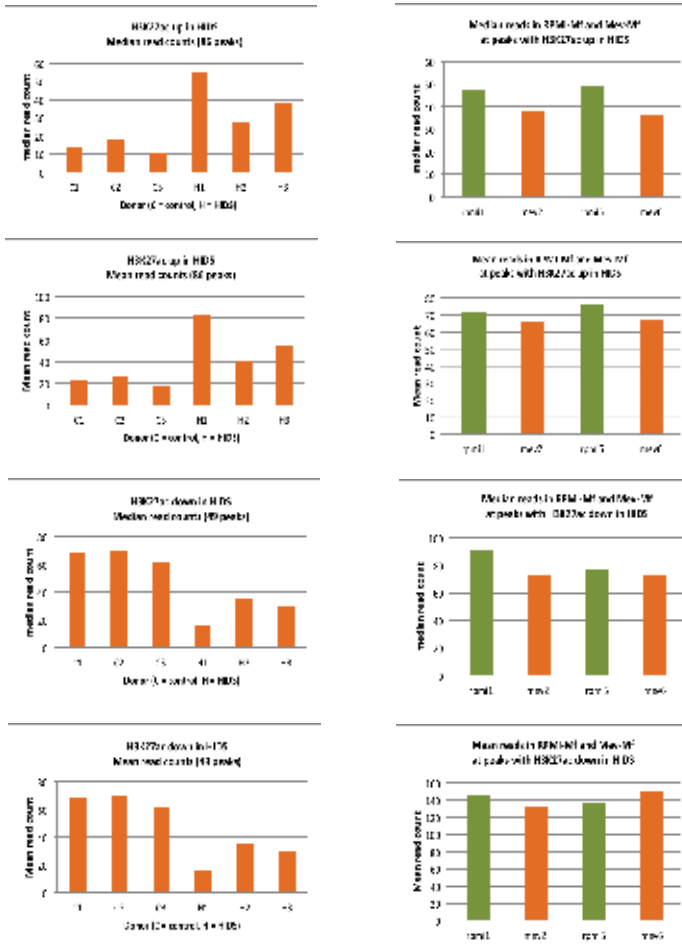
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## SUPPLEMENTARY FIGURES

**Figure S1** - HIDS expression analysis. Heatmap of individual donors for genes that show LPS responses in controls and HIDS patients. Monocytes were either unstimulated (=baseline) or stimulated for 24h with RPMI/LPS.



**Figure S2** – Comparison of H3K27ac peaks in HIDS monocytes versus mevalonate-trained macrophages. The left hand side graphs display the median and mean read counts of the H3K27ac peaks that were significantly up or down in HIDS patient monocytes. At the right hand side graphs are display of median and mean read counts at the same H3K27ac peaks. Table S1. Up- and down-regulated peaks of H3K27ac in mevalonate-trained macrophages and a comparison between mevalonate and beta-glucan trained macrophages.



**Table S2.** RNAseq analysis of unstimulated or 24h RPMI- or LPS-stimulated monocytes from HIDS patients and controls (n=3). (data not shown)

**Table S3.** Whole-genome assessment of the histone mark H3K27ac by ChIP-sequencing from HIDS patients and controls (n=3). Comparison between histone acetylation of HIDS patients monocytes and mevalonate trained macrophages. (data not shown)







# CHAPTER 8

## LONG-TERM ACTIVATION OF THE INNATE IMMUNE SYSTEM IN ATHEROSCLEROSIS

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## ABSTRACT

Efforts to reverse the pathologic consequences of vulnerable plaques are often stymied by the complex treatment resistant pro-inflammatory environment within the plaque. This suggests that pro-atherogenic stimuli, such as LDL cholesterol and high fat diets may impart longer lived signals on (innate) immune cells that persist even after reversing the pro-atherogenic stimuli. Recently, a series of studies challenged the traditional immunological paradigm that innate immune cells cannot display memory characteristics. Epigenetic reprogramming in these myeloid cell subsets, after exposure to certain stimuli, has been shown to alter the expression of genes upon re-exposure. This phenomenon has been termed trained innate immunity or innate immune memory. The changed responses of 'trained' innate immune cells can confer nonspecific protection from secondary infections, suggesting that innate immune memory has likely evolved as an ancient mechanism to protect against pathogens. However, dysregulated processes of immunological imprinting mediated by trained innate immunity may also be detrimental under certain conditions as the resulting exaggerated immune responses could contribute to autoimmune and inflammatory diseases, such as atherosclerosis. Pro-atherogenic stimuli most likely cause epigenetic modifications that persist for prolonged time periods even after the initial stimulus has been removed. In this review we discuss the concept of trained innate immunity in the context of a hyperlipidemic environment and atherosclerosis. According to this idea the epigenome of myeloid (progenitor) cells is presumably modified for prolonged periods of time, which, in turn, could evoke a condition of continuous immune cell over-activation.

## ATHEROSCLEROSIS, A PERSISTENT INFLAMMATORY DISEASE

Cardiovascular diseases (CVD), including stroke, and myocardial infarction, are the leading cause of mortality worldwide and together are responsible for approximately 17 million deaths per year [1]. The vast majority of cardiovascular events are caused by the rupture or erosion of atherosclerotic plaques in the arterial wall and the subsequent formation of an occluding thrombus. Traditionally, atherosclerosis is regarded as a disease of the developed Western Society, driven by the major classical risk factors dyslipoproteinemia, hypertension, diabetes, obesity, smoking, and a sedentary lifestyle. Interestingly, however, the development of atherosclerosis is not restricted to patients with a Western lifestyle. In a recent study, CT-scanning revealed signs of atherosclerosis in a third of mummies from several ancient populations, even in non-smoking populations that had an almost entirely marine diet [2]. These observations fit the paradigm shift in the early nineties that atherosclerosis is more than simply a vascular cholesterol storage disease, and that chronic low-grade vascular inflammation triggered by the deposited material could contribute to atherogenesis [3]. The inflammatory nature of atherosclerotic plaques was already recognized in the nineteenth century by Virchow [4], who wrote: “In some, particularly violent cases the softening manifests itself even in the arteries not as the consequence of a really fatty process, but as a direct product of inflammation”.

In the last ten years, several novel non-traditional risk factors for atherosclerosis have been identified that are all associated with activation of the immune system. These include chronic inflammatory diseases such as rheumatoid arthritis, gout, psoriatic arthritis, and ankylosing spondylitis, as well as infections with bacteria or viruses, such as *Chlamydia pneumoniae*, *Porphyromonas gingivalis* and human immunodeficiency virus (HIV) [5]. In addition, acute cardiovascular events occur more frequently in the weeks following an acute infection, such as pneumonia [6]. Recently, also cardiovascular events itself, such as myocardial infarction [7] or stroke were described to drive subsequent acceleration of atherosclerosis throughout the vascular system [8]. Finally, recent experimental evidence indicates that changes in diet and in dietary composition are able to directly influence lymphoid organs, thus profoundly and continuously influencing immune responses and the development of inflammatory and autoimmune diseases [9].

Although it is now well established that inflammation plays an essential role in the initiation, progression, and destabilization of atherosclerotic plaques, the mechanisms that drive the persistent non-resolving inflammation in the vessel wall remain incompletely understood. Macrophages are the most abundant inflammatory cells present in atherosclerotic plaques.

Cells of the adaptive immune system such as T and B lymphocytes are also present, but in lower numbers. Evidence is rapidly accumulating that innate immune cells can adopt a persistent pro-inflammatory phenotype after brief exposure to a variety of stimuli, a phenomenon that has been termed ‘trained innate immunity’ [10].

In this review, we discuss the concept that trained immunity contributes to the development of atherosclerosis, both in the setting of traditional cardiovascular risk factors, and in the setting of non-traditional risk factors, such as acute and chronic infections, and non-infectious chronic inflammatory disorders. In addition, it might well be that elevated dietary fats and consequently an alteration of the gut microbiota, impact immune homeostasis (dysbalance between inflammatory and tolerogenic processes), thus inducing a long-term epigenetic reprogramming of innate immune cells.

## THE INNATE IMMUNE SYSTEM AND ATHEROSCLEROSIS

Within atherosclerotic plaques, macrophages are the most abundant subset of leukocytes. Arterial resident macrophages are derived from embryonic CX3CR1<sup>+</sup> precursors and from bone marrow-derived monocytes that colonize the tissue immediately after birth [11, 12]. During atherogenesis, arterial plaque macrophages are sustained by local proliferation, but also by recruitment of Ly6C<sup>high</sup> monocytes [13]. Monocytes and macrophages are critically promoting the initiation, progression, and destabilization of atherosclerotic plaques by several mechanisms [14, 15]. In the early stages of atherosclerosis, macrophages contribute to the clearance of reactive lesional oxidized low-density lipoprotein (oxLDL) particles through scavenger receptor A (SR-A) and CD36 mediated uptake. In later stages, macrophages are impaired in their phagocytic functionality due to intracellular accumulation and defective efflux of oxLDL. Foam cell macrophages undergo apoptosis due to cellular stress and inflammatory responses, a process, which ultimately contributes to the formation of the pro-thrombotic necrotic core that characterizes mature atherosclerotic plaques. Damage Associated Molecular Patterns (DAMPs), which accumulate in the atherosclerotic plaques, represent a source of endogenous danger ligands that can further activate macrophages by binding to specific Pattern Recognition Receptors (PRR), such as membrane-bound Toll Like Receptors (TLR), SR, and intracellular NOD-like receptors (NLRs) [16]. Multiple pro-inflammatory triggers could hence cause an inflammatory milieu in the plaque. In addition, even the phase transition of cholesterol can cause inflammation as cholesterol crystals can activate the Nlrp3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome, triggering the secretion of pro-inflammatory IL-1 $\beta$  and IL-18 [17]. Finally, lesional macrophages contribute to the remodeling of atherosclerotic plaques into a rupture-prone unstable phenotype by the production of proteases, such as matrix metalloproteinases [18]. Overall, plaque macrophages

display a marked phenotypic heterogeneity [19], which is dictated by the composition of the local DAMPs that are present in the specific environment during different stages of plaque development.

Several studies have shown that hypercholesterolemia considerably increases the number of circulating Ly6C<sup>high</sup> pro-inflammatory monocytes in mice (or the CD14<sup>++</sup>CD16<sup>-</sup>/ CD14<sup>++</sup>CD16<sup>+</sup> equivalent in humans), thereby accelerating atherosclerosis [20, 21]. Additionally, an acute myocardial infarction, stroke or sepsis can accelerate subsequent atherosclerosis by inducing monocytosis [7, 22]. Which factors are driving monocytosis in the context of a hypercholesterolemic environment remains to be fully resolved. Besides, although the adverse contribution of myeloid cells in atherosclerosis is generally accepted, it is still an open question why a strong pro-inflammatory response within the arterial wall fails to be resolved even after reducing potential inflammatory triggers. Recent studies have illuminated the mechanisms underlying the hypercholesterolemia-induced myelopoiesis and a shifting towards the pro-inflammatory Ly6C<sup>high</sup> monocytes [23, 24]. Hitherto, the debate has primarily focused on the link between cholesterol accumulation within the plaque macrophage and local ongoing inflammatory processes. However, myeloid priming and reprogramming under hypercholesterolemic conditions can occur already within the bone marrow, due to an overload of cholesterol and defective efflux pathways in hematopoietic stem and myeloid precursor cells. Animal studies have shown that cholesterol efflux pathways in hematopoietic stem cells (HSC) are mediated by apolipoprotein E (ApoE), ATP-binding cassette transporter A1 (ABCA1) and ABCG1 and these processes are controlling HSC expansion in the bone marrow. Transplantation of ABCA1<sup>-/-</sup> ABCG1<sup>-/-</sup> bone marrow into Ldlr<sup>-/-</sup> mice fed a Western type diet, as well as studies in ApoE<sup>-/-</sup> mice fed a Western type diet, induced dramatic leukocytosis (monocytosis and neutrophilia), which could be reversed by overexpression of the transgenic human Apo-AI [25-27]. The proliferative response in HSC was favored by increased cholesterol storage into the plasma membrane (due to impaired efflux), which resulted in increased expression of the proliferative cytokine receptors to IL-3, IL-5 and GM-CSF. Recent evidence suggests that Western type diet feeding also induces epigenetic modifications within HSC and myeloid progenitor subsets, potentially provoking increased myelopoiesis [28, 29]. Van Kampen et al. [29] revealed in competitive bone marrow transplantation experiments using Ldlr<sup>-/-</sup> recipients fed a chow diet that donor bone marrow from Western type diet fed animals markedly increased myeloid progenitor proliferation compared to donor bone marrow from chow fed animals. Bone marrow from Western type diet fed mice showed hypomethylation in CpG-regions in the genes encoding PU.1 and IRF8, key regulators of monocyte proliferation and macrophage differentiation. Consistently, blood leukocyte numbers were significantly increased as a result of increased circulating monocytes, along with the presence of a

more activated splenic macrophage phenotype and increased plaque sizes. In agreement with these data, Seijkens and colleagues [28] demonstrated in a competitive bone marrow transplantation study (transfer of normocholesterolemic bone marrow cells into either chow or high fat diet fed *Ldlr*<sup>-/-</sup> recipients) that a hypercholesterolemic BM microenvironment induced loss of HSC quiescence, characterized by increased expression of cyclin B1, C1 and D1, and skewed HSC development towards myeloid lineages, especially towards granulocytes and Ly6C<sup>high</sup> monocytes. HFD priming of bone marrow HSC remained even after transfer into normocholesterolemic mice and was associated with reduced H3K9/14 acetylation at the promoter of the retinoblastoma (RB) gene (involved in the control of excessive cell growth). In summary, these studies shed new light on the causality between hypercholesterolemia, altered epigenetic patterning in HSC and myeloid progenitors, and increased susceptibility to atherosclerosis.

Indeed, the concept of epigenetic regulation is being increasingly acknowledged as an important contributor in the pathogenesis of atherosclerosis and other metabolic diseases. Research within the last decade has provided an essential link between pro-atherogenic factors (amongst others diet, disturbed blood flow patterns, dyslipoproteinemia, hyperglycemia and microbiome) and reprogramming of the epigenome (altered DNA methylation and histone modifications) in various cell types such as leukocytes, vascular endothelial cells (EC) and vascular smooth muscle cells (vSMC) [30, 31].

## TRAINED INNATE IMMUNITY

Although cells of the innate immune system are traditionally considered incapable of building an immunological memory, plants and invertebrates that lack an adaptive immune system are protected against reinfection with pathogens, suggesting that the response of the innate immune system can be modified by previous encounters [10]. Trained innate immune responses were confirmed in human monocytes *ex vivo* by showing that monocytes can adopt a long-term pro-inflammatory phenotype after brief exposure to various micro-organisms or microbial products, including the bacille Calmette-Guérin (BCG) vaccine, *Candida albicans*, or its cell wall component  $\beta$ -glucan [32, 33]. This phenomenon could also be shown in humans *in vivo* by demonstrating that BCG vaccination in healthy human subjects induced an increased production of pro-inflammatory cytokines when isolated monocytes were exposed *ex vivo* to various unrelated microbial metabolites [32]. This enhanced pro-inflammatory phenotype of the monocyte could be detected even three months after the first vaccination suggesting that trained innate immune responses can persist for relatively long periods. The powerful immunologic effects of this phenomenon are illustrated by the findings in scid

mice, which lack T and B lymphocytes, where BCG vaccination provided robust protection against a subsequent lethal dose of *C. albicans*. In humans, BCG vaccination prior to influenza vaccination results in a more pronounced increase and accelerated induction of functional antibody responses against an influenza vaccine [34]

Importantly, trained innate immunity cannot only be induced by microbial products, but most likely also by metabolites that are relevant in the development of metabolic diseases and its complications. In the context of atherosclerosis, innate immune training might occur in a high fat and high cholesterol environment occurring within the plaque and potentially elsewhere, such as in the gut, liver or bone marrow. We postulate that long-term epigenetically reprogrammed myeloid precursor cells that are characterized by a hyper-inflammatory phenotype may contribute to a sustained disease progression [35] (Figure 1).

## HUMAN IN VITRO STUDIES

In an in vitro model in human isolated monocytes, we found that trained innate immunity can be induced by modified LDL (oxLDL as well as acetylated LDL), but not by native LDL [36]. Twenty-four hour exposure of human monocytes to low concentrations of oxLDL resulted in

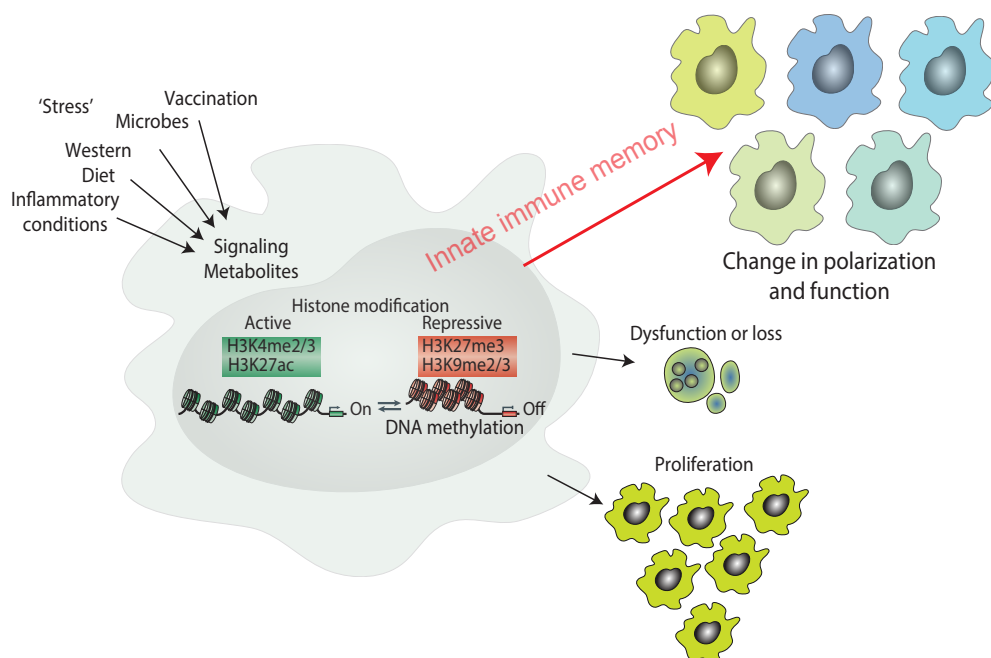


Figure 1: Cellular stressors can influence the epigenetic landscape of innate immune cells. Depending on the chromatin modification the outcomes lead to different immune responses of the cells towards secondary stimuli, a concept that has been termed 'innate immune memory', with alternative fates being polarization, cell death or increased proliferation.



an increased production of pro-atherogenic cytokines and chemokines (including IL-6, TNF $\alpha$ , IL-8, and MCP-1) upon re-stimulation of the cells 7 days later with TLR4 or TLR2 agonists. Additionally, IL-18, MMP2, and MMP9 mRNA-expression was significantly higher in the trained monocytes and increased foam cell formation with higher expression of the scavenger receptors CD36 and SR-A and decreased expression of the cholesterol efflux transporters ABCA1 and ABCG1 was found. Importantly, oxLDL-induced trained macrophages showed no significant differences in expression of TLR4, TLR2 and nitric oxide synthase ('M1' markers); arginase1 and CD163 ('M2' markers); and HLA-DR (human leukocyte antigen-DR), DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin), and CD83 expression (DC markers). These data illustrate that the trained innate immune phenotype does not merely represent skewing of the cells into the classical 'M1' but rather represents a unique pro-inflammatory phenotype. Pathway analysis revealed that oxLDL-induced training is dependent on TLR2 and TLR4 activation, and activation of extracellular regulated kinase (ERK) and phosphoinositide 3 kinase (PI3K) [36].

## IN VIVO STUDIES

Corroborating the human in vitro data, it has previously been reported that subcutaneous immunization of cholesterol-fed New Zealand White rabbits with a human dose of BCG enhanced peripheral leukocyte activation, aortic monocyte recruitment and atherogenesis [37]. Although this finding fits within the concept that trained innate immunity contributes to atherogenesis, a contributory role for the adaptive immune system cannot be excluded in these experiments. In addition, several papers provide evidence that glucose can also induce a persistent immunological memory in various cell types that are relevant in the context of atherosclerosis, including EC, vSMC, and monocytes. In a mouse model of diabetes, long-term activation of several key inflammatory genes in isolated vSMC was described, which correlated with increased H3K4 methylation and suppressed H3K9 di- and tri-methylation at the nuclear factor  $\kappa$ B (NF- $\kappa$ B) subunit p65 promoter, which persisted even after ex vivo cell culturing [38, 39]. Also, brief exposure to high-glucose medium in cultured EC induced a persistent pro-inflammatory phenotype, even after reversal to normoglycemic conditions. This was associated with enrichment of the activating mark H3K4me1 in the NF- $\kappa$ B subunit p65 [40]. These results highlight the surprisingly long-lasting effects that short-term hyperglycemic spikes can execute on vascular cells, suggesting the idea of memory formation as a result of methyl-writing and methyl-erasing histone modifications [41]. In line, the concept of epigenetically mediated metabolic memory was recently also illustrated in patients from the Diabetes Control and Complications Trials (DCCT). Isolated monocytes from patients without diabetic microvascular complications, that had received intensive diabetes regulation in the

past, showed overall hyperacetylation of H3K9 compared to patients with microvascular complications who had received less-intensive diabetes regulation in the past [42]. A link to glucose induced alterations in the bone marrow niche has recently been illustrated by the observation that a high-glucose environment induces stable intrinsic pro-inflammatory changes in HSC in mice [43].

Further in vitro and in vivo studies are required to corroborate the concept of trained innate immunity due to persistent epigenetic changes in response to ‘epigenetically toxic’ nutrient and/or microbial metabolites such as high fat, high cholesterol or high glucose in the context of metabolic diseases and its complications including atherosclerosis. Nevertheless, one supporting issue in the theory of epigenetic reprogramming and development of a trained memory is the trans-generational transmission of epigenetic traits, independently of genetically inherited risk factors [44]. In this regard, several studies in humans and experimental animal models have shown that parental adverse diet feeding (under- or over-nutrition) not only influences the individuals environment, but can also influence the metabolic phenotype in the offspring thereby raising metabolic and cardiovascular disease susceptibility [45-48]. However, dissecting the effects induced by an unfavorable intra uterine environment to which fetus is exposed to during gestation from direct effects on the parental germ line remains a challenging task. To determine whether epigenetic information can be transmitted through the germ line, multiple animal studies have tested the question of whether the offspring responded to changed paternal diet conditions. Indeed, paternal low-protein diet induced an up-regulation of genes involved in lipid and cholesterol biosynthesis in both male and female offspring, which was associated with a changed DNA methylation pattern in enhancer regions of peroxisome proliferator-activated receptors (PPAR)  $\alpha$  [49]. Likewise, chronic HFD feeding of male rats led to altered expression of genes involved in glucose metabolism due to a changed DNA methylation pattern, subsequently impairing glucose tolerance as well as insulin sensitivity in female offspring [50]. A recently published study demonstrates that a high-fat/high-fructose diet, starting at young age, leads to the development of obesity and to the progression of metabolic syndrome, accompanied by altered DNA methylation patterns in male and female Wistar rats [51]. HFD feeding was shown to affect 5-mC levels differently in both genders with a 20% reduction in males and a 15% increase in females. Interestingly, DNA methylation patterns were modified as well in the F1 generation, thus proposing that HFD-induced epigenetic modifications – as mediator of metabolic memory - can be transmitted to the next generation, subsequently increasing the susceptibility for disease development. Three recent studies support the idea of epigenetic inheritance by the paternal germ line in demonstrating that nutritional change can directly remodel the epigenetic signature of spermatozoa. A comprehensive profiling of the sperm epigenome

showed that mal-nutrition as well as over-nutrition altered small RNA levels, including tRNA fragments, and DNA methylation patterns, indicating that sperm (as the direct transducer of genetic information) is vulnerable to environmental changes [52-54]. Whether epigenetic changes in the myeloid lineage can be transmitted to offspring remains unanswered yet.

## THE POTENTIAL MECHANISMS LEADING TO TRAINED INNATE IMMUNITY

The more prone pro-inflammatory phenotype that characterizes trained innate immune cells is critically dependent on a complex integration of epigenetic reprogramming and a change in the intracellular immuno-metabolism.

### Epigenetic reprogramming

Epigenetic modulation denotes the regulation of gene transcription independent of the DNA sequence. Epigenetic regulation can occur at the level of DNA methylation, histone modifications, or involves RNA-based mechanisms. Whereas DNA methylation is associated with a condensed chromatin structure that prevents binding of transcription factors and gene silencing, histone acetylation is associated with activation of gene transcription. Histone methylation can either activate or repress gene transcription, depending on the exact location of the methylated lysine residue and on the number of bound methyl groups (mono-, di-, or trimethylation). Macrophage differentiation, polarization, and activation can all be influenced by epigenetic reprogramming, which is reviewed in detail elsewhere [55-57].

A distinguishing hallmark of a trained innate immune cell is its ability to exhibit a quantitatively and qualitatively modified- usually more intense- inflammatory response upon re-challenge with danger signals that differ from the priming stimulus. The underlying molecular basis has thus far only partially been resolved and involves changes in chromatin organization (loosening and stringent accessibility to the transcriptional machinery), but might also comprise alterations in DNA methylation and RNA functionality (miRNAs, lncRNAs, tRNAs) caused by a certain primary stimulus [58, 59]. Two recent studies support the concept of trained innate immunity in that they evidenced latent or de novo enhancer formation in stimulated macrophages [60, 61]. TLR4-induced NF- $\kappa$ B activation in macrophages resulted in binding of the transcription factors (TF) PU.1/ C/EBP to an appropriate enhancer element. TF binding was accompanied by recruitment and binding of the NF- $\kappa$ B-p65 subunit and histone acetyltransferases, and subsequent RNA polymerase II binding and elongation. Finally, histone methyltransferases MLL1, MLL2/4 and MLL3 were recruited, being responsible for H3K4 monomethylation and fully active enhancer formation. Notably, de novo formed enhancer elements remained stable (H3K4me1) upon loss of stimulus, and re-stimulation of macrophages resulted in faster and stronger responses.

For oxLDL-induced trained immunity in isolated human monocytes, epigenetic reprogramming at the level of histone methylation is crucial. Brief stimulation of human monocytes with oxLDL leads to the acquirement of a 'trained innate memory', which was associated with a

pro-atherogenic and pro-inflammatory phenotype, and was characterized by enriched H3K4 trimethylation – a prototypical long-term activating epigenetic mark - on the promoters of pro-inflammatory cytokines and chemokines [36]. Moreover, trained innate immunity was completely prevented by co-incubation with a nonspecific histone methyltransferase inhibitor.

For training with  $\beta$ -glucan, an unbiased genome-wide experimental approach revealed changes in the activating H3K4me3 levels on promoters, H3K4me1 levels on enhancers, and H3K27ac levels on promoters and enhancers. Indeed, most dynamic promoters showed an enrichment of these marks [62]. Moreover, pathway analysis of the promoters that were potentiated by  $\beta$ -glucan identified several innate immune and signaling pathways upregulated in trained cells that are responsible for the induction of trained immunity.

### Immuno-metabolism and epigenetic programming

Epigenetic modifications are indispensable in cell reprogramming, however, it still remains an open question which cellular processes act to initiate or maintain these modifications. Increasing evidence supports a close connection between systemic and cellular metabolic processes and epigenetic reprogramming with a crucial role for cellular metabolites, e.g. S-adenosylmethionine (SAM), flavin adenine dinucleotide (FAD), b-hydroxybutyrate (b-OHB), acetyl-CoA, NAD<sup>+</sup>,  $\alpha$ -ketoglutarate, and adenosine triphosphate (ATP) as co-modifiers for epigenetic ‘writing’ (DNA and histone methyltransferases, lysine acetyltransferases) and ‘erasing’ (DNA demethylases, histone deacetylases, lysine demethylases) enzymes [63, 64]. The metabolic state of an immune cell represents a highly dynamic process and changes between homeostasis and immune activation (tolerance versus inflammation), all of which has been documented in different macrophage phenotypes. Generally, resting macrophages and the ‘M2’ phenotype (involved in tissue repair and wound healing) utilize energetically efficient processes including an intact Krebs cycle with ATP generation via oxidative phosphorylation. Upon immune cell activation and a switch towards the ‘M1’ phenotype there is a rapid shift towards aerobic glycolysis, which enables de novo lipogenesis, cholesterol and amino acid synthesis for subsequent fast cell division and growth, and an increased production of inflammatory mediators (eicosanoids, IL-1b, reactive oxygen species) [65, 66]. Hence, the cellular transcription machinery and its chromatin associated proteins process cellular metabolic signals to regulate gene expression.

The importance of immuno-metabolism in macrophage polarization and reprogramming suggests that similar mechanisms take place during the long-term acquirement of a trained innate memory phenotype. Indeed, a shift from oxidative phosphorylation towards aerobic glycolysis through the AKT-mTOR-HIF-1a pathway, accompanied by changes in histone marks

such as H3K4me1, H3K4me3 and H3K27ac, has recently been described to be important for training of human monocytes with  $\beta$ -glucan. Pharmacological inhibitors of glycolysis, such as 2-deoxy-D-glucose and inhibitors of the mTOR pathway, such as metformin, prevented  $\beta$ -glucan-induced trained innate immunity [62, 67]. LPS-priming induced a shift towards aerobic glycolysis in murine macrophages, which resulted in the accumulation of the Krebs cycle intermediate succinate, which itself induced the expression of the inflammatory cytokine IL-1b through the transcription factor HIF-1a [68]. These studies emphasize that Krebs cycle metabolites (such as succinate,  $\alpha$ -ketoglutarate or NAD<sup>+</sup>) are important in the induction or inhibition of histone-modifying enzymes, thus inducing long-term changes in the monocyte or macrophage phenotype.

How immuno-metabolism is altered under conditions of hyperlipidemia and which metabolites potentially contribute to the establishment of a trained myeloid (precursor) cell phenotype in atherosclerosis is still ill-defined. Transcription factors such as sterol regulatory element binding protein (SREBP; regulation of cholesterol biosynthesis and uptake), liver X receptor (LXR; regulation of cholesterol elimination and inflammation) and PPARs (regulation of fatty acid oxidation/ energy homeostasis, lipid metabolism and inflammation) strictly control the balance between systemic and cellular lipid homeostasis by regulating genes involved in lipid uptake, storage, efflux, de novo lipid biosynthesis and catabolism, as well as inflammatory processes. Excessive lipid or cholesterol uptake by immune cells has been shown to favor inflammatory processes, which accounts for the exacerbated outcome of diseases associated with chronic metabolic inflammation (obesity, atherosclerosis). For example, overload of modified lipoproteins (oxLDL) in liver Kupffer cells induces a continuous state of intracellular stress and promotes a switch towards the pro-inflammatory 'M1' phenotype [69]. Hyperlipidemia also modulates the immune system by enhancing bone marrow and extramedullary hematopoiesis through epigenetic reprogramming of genes crucially involved in monocyte proliferation and differentiation [29]. Somewhat unexpectedly, Spann and colleagues have recently shown that peritoneal macrophages isolated from Western type diet fed *Ldlr*<sup>-/-</sup> mice exhibited an overall reduction in pro-inflammatory gene expression compared with macrophages from chow fed mice, which was related to the accumulation of desmosterol, an intermediate metabolite in the cholesterol biosynthesis pathway and activator of LXRs [70]. Desmosterol accumulation in peritoneal foam cell macrophages might be explained by impaired functionality of desmosterol reductase, the metabolic enzyme for desmosterol degradation. Together, these data clearly illustrate that firstly a tight interplay between the different lipid sensing nuclear receptors is important to balance intracellular lipid and cholesterol homeostasis and to control immune signaling pathways, such as NF- $\kappa$ B, or Nlrp3. Secondly, the specific environment crucially contributes to metabolic processes,

which critically influences inflammatory responses in different myeloid subsets.

### NUTRITIONAL CONTROL OF IMMUNITY

Diet and its components can activate cells of the innate immune system, thus profoundly influencing immune responses, and potentially inducing long-term epigenetic modifications and a 'trained innate memory', especially within the gut resident myeloid subsets. In line, the gut microbiota plays an important role in the development and maintenance of the immune system. The microbiota affects immune cell metabolism and function either through metabolites derived from its enzymatic machinery, such as short chain fatty acids (SCFA), bile acid, or trimethylamine (TMA), or through particular microbial molecules, such as innate immune receptor activating ligands [71-73]. Maternal and early-life dietary habits already play an important role in the development of the immune system, and the prevention of immunological disorders later in life [74]. The composition (quantity and quality) of free fatty acids and lipids (and their oxidative metabolites) in the diet is crucial in maintaining immune cell homeostasis and influences both innate and adaptive immune cell responses. Changes in dietary habits such as increased intake of energy-dense processed food enriched in sugars, fat, phosphatidylcholine (PC) and L-carnitine instead of nutrient-rich foods, induce a shift from commensal to more pathogenic bacterial strains within the gut.

These changes in enteric microbiome composition correlate with an increased incidence of immunological disorders, such as asthma, cancer, allergies, autoimmune and cardiovascular diseases [75, 76]. In the contrary, diets enriched in complex carbohydrates and high fibers, such as found in vegetables, fruits and fish, promote gut homeostasis. These diets can beneficially influence amongst others the production of antimicrobial peptides and mucus, maintenance of epithelial integrity and tissue repair, production of anti-inflammatory cytokines and maintenance of immune tolerance. Mechanistically, the microbial enzyme machinery can process these fiber-rich food into SCFA, including n-butyrate, propionate and acetate, and can influence the production of other end products including idole-3-aldehyde, niacin and omega-3 fatty acids. Besides their function as energy sources, these metabolites control gut homeostasis by signaling through different receptors, e.g. the Aryl hydrocarbon receptor (AhR) and G-protein coupled receptors (GPCR), expressed on innate lymphoid cells, innate immune cells and Tregs. Food-induced epigenetic reprogramming in innate immune cells has been supported by a recent study, in which the SCFA n-butyrate led to the down-regulation of LPS-induced pro-inflammatory mediators, including nitric oxide, IL-6, and IL-12 [77]. These effects were independent of TLR-signaling or activation of GPCRs, but were due to inhibition of histone deacetylases by n-butyrate. Overall, this rendered lamina propria

macrophages hypo-responsive and maintained a tolerogenic environment.

Western type diets are enriched in poly-unsaturated fatty acids, associated with dysbiosis, a state of low-grade chronic inflammation in the intestines through activation of NF- $\kappa$ B and the Nlrp3 inflammasome, and increased intestinal permeability resulting in endotoxemia [78]. Indeed, studies in humans and animal models have shown that an accumulation of lipids and cholesterol, and an impaired lipid clearance in myeloid cells are associated with worse septic shock outcome due to increased TLR2 and 4 activation [79]. A systemic-wide metabolomics approach has recently shown that metabolites (choline, TMA, betaine) derived from the dietary phospholipid PC or L-carnitine represent risk factors for cardiovascular diseases. Indeed, increased systemic levels of these metabolites correlate with more aggravated CVD phenotype [80, 81]. Consistent with these observations, ApoE<sup>-/-</sup> mice fed a diet enriched in PC or L-carnitine, exhibited worsened atherosclerotic disease outcome [80, 81]. PCs and L-carnitine, enriched in red meat, eggs, milk and certain fish, are processed by the gut microbial enzymatic machinery to choline and TMA, which is further metabolized to TMA n-oxide (TMAO) by the hepatic flavin mono-oxygenase (FMO). TMAO has been shown to increase the expression of the scavenger receptors CD36 and SR-A1 on macrophages, subsequently promoting foam cell formation. Additionally, it adversely affects reverse cholesterol transport; both processes that promote atherosclerosis.

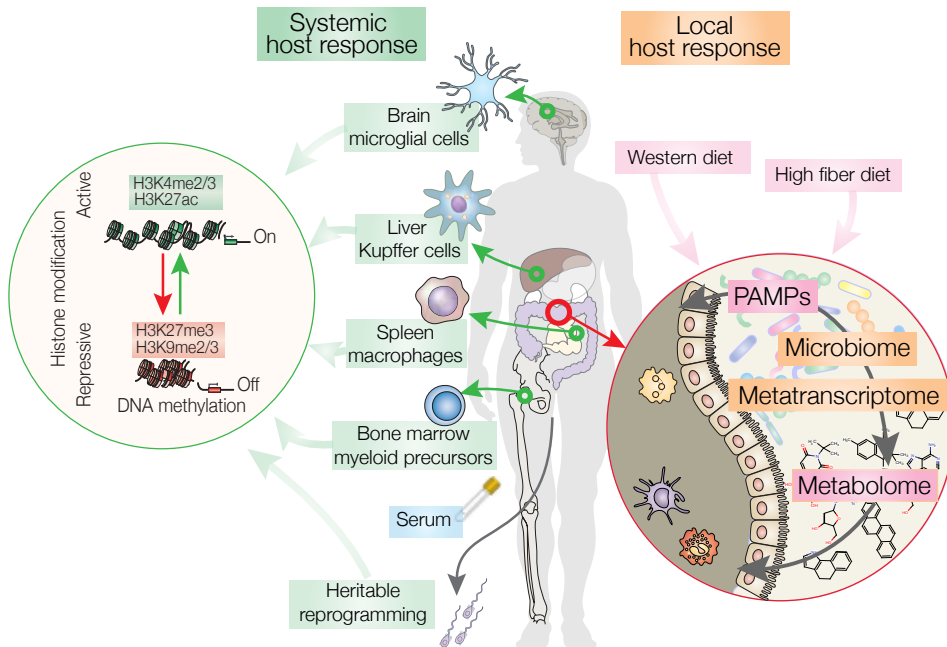
The above-summarized data illustrate the complex network between food composition, the microbiota and host's metabolism linked to immune homeostasis. Consequently, a balanced diet, rich in fibers, keeps a balanced microbiota and an intact intestinal barrier function, which is a requirement for a regulated metabolism and immune homeostasis (Figure 2). It still remains to be resolved how systemic to cellular immune-metabolic crosstalk, impacted by the gut microbial composition, is working, and how this can lead to a long-term epigenetic reprogramming.

## THERAPEUTIC INTERVENTIONS IN ATHEROSCLEROSIS AND POTENTIAL CLINICAL RELEVANCE

### Targeting LDL/ cholesterol levels and inflammation in atherosclerosis

So far, statins are still used as the main drug treatment in CVDs to lower plasma cholesterol levels, thus decreasing disease burden. However, despite the LDL lowering effect up to > 50%, a significant residual burden of CVDs remains. Alternatively, ApoA-I containing high density lipoprotein (HDL) is being developed as a therapeutic agent to remove excess cholesterol from





**Figure 2:** Schematic representation of the hypothesis that diet can have long lasting effect on the systemic host response via epigenetic reprogramming by microbiome-derived PAMPs and metabolites.

peripheral tissue, and to dampen inflammation [82-85]. Studies in different animal models as well as in humans have shown the beneficial effect of HDL administration in reducing atherosclerotic plaque size [86-88]. Notwithstanding, infused HDL in two randomized controlled clinical trials in human have not reached the expected beneficial effects on atherosclerosis [89, 90].

In the last years scientists have searched for more intelligent ways of drug delivery, but also for novel therapeutic targets to dampen atherosclerotic lesion progression. The use of nanoparticles seems to be an attractive therapeutic tool to deliver anti-inflammatory drugs to specific cell types with the aim to impair immune actions in certain cell subsets e.g. restricting myeloid cell differentiation and migration of pro-inflammatory Ly6C<sup>high</sup> monocytes to the plaque, dampening plaque macrophage immune signaling and increasing macrophage efflux. Some recent preclinical studies have successfully been proposed [91, 92].

#### Epigenetic remodeling as a potential therapeutic approach for CVD

The recent insights into possible mechanistic links between epigenetic cellular reprogramming and pathophysiology of atherosclerosis could lead to novel strategies to lower CVD risk [93,

94]. Moreover, epigenetic modifications (DNA methylation pattern, modified histone marks) might evolve into useful predictors for disease stage [95] and have recently been linked to atherosclerosis in humans [30, 96]. Wierda and colleagues evaluated global H3K27me3 levels, as well as levels of histone-modifying enzymes (EZH2, BMI1 and JMJD3) in vessels representing different stages of atherosclerotic plaque development. Overall, they notified a global decrease in H3K27me3 in the media of more advanced atherosclerotic plaques, which might reflect the dynamic pattern of vSMC differentiation and proliferation associated with atherosclerotic disease [30].

Co-modifying metabolites as well as ‘writing’ and ‘erasing’ enzymes are amenable to pharmacological modulations, and could potentially turn into novel therapeutic targets. A few nonselective HDAC inhibitors are already used in regular patient care in cancer treatment [97]. In addition, nonselective HDAC inhibitors have shown beneficial effects in animal models for arthritis, septic shock and inflammatory bowel disease [98]. Preclinical studies in mice support the idea that ‘epigenetic drugs’ may also be a potential tool in the treatment of atherosclerosis [99]. Administration of the nonselective HDAC inhibitor trichostatin A (a lysine deacetylase inhibitor) into *Ldlr*<sup>-/-</sup> mice induced a more exacerbated neointima formation by increasing CD36 mRNA, protein and surface expression levels thus increasing macrophage foam cell formation.

Histone demethylases can also induce a pro-inflammatory macrophage phenotype. For example, *Jmjd3*, *UTX* and *Uty* (which are H3K27 demethylases) are induced upon TLR engagement and are linked to the expression of inflammatory genes [100]. Targeting of *Jmjd3* and *Utx* H3K27 demethylases with small molecule inhibitors impaired inflammatory responses in human primary macrophages, which is of high pharmacological interest [101]. However, *Jmjd3* also plays an important role in the ‘M2’ polarization in the context of helminth infection, illustrating its dual role dependent on stimulus and environmental context [102]. Selective HDAC inhibitors may be used to dampen inflammatory responses within plaque macrophages. Three recent studies illuminated a functional role for certain HDACs in the context of atherosclerosis. Myeloid deletion of HDAC3, a histone deacetylase which promotes ‘M1’ polarization [103], was shown to augment lesion size, but lesions showed a more stable phenotype characterized by a collagen-rich fibrous cap, reduced lipid content and reduced numbers of plaque macrophages. The more stable plaque phenotype was associated with increased collagen formation by vSMC due to increased TGF- $\beta$  release by HDAC3 deleted myeloid cells [104]. In addition, a genome-wide association study illustrated the association between HDAC9 modifications and atherosclerosis development. In fact, certain HDAC9 transcripts are more related to coronary artery disease susceptibility [105].

Consistent with these data, Cao and colleagues revealed a correlation between HDAC9 deletion in bone marrow cells and limited atherosclerosis development in the *Ldlr*<sup>-/-</sup> mouse atherosclerosis model. HDAC9 deletion in macrophages was linked to an increased expression of the cholesterol efflux transporters ABCA1 and ABCG1, reduced inflammation, and a switch towards the ant-inflammatory 'M2' phenotype [106].

The above-mentioned studies support the impact of epigenetic remodeling in the pathophysiology of atherosclerosis, thus encouraging the development of epigenetic tools for beneficial lesion remodeling. Nevertheless, it has to be noted that different isoforms of histone modifying enzymes might have opposing effects within the same cells, but also in different cell types. Additionally, enzymes might function differently in healthy versus acute versus chronic disease stages of atherosclerosis. A tremendous challenge within the next years will be to better understand epigenetics in the pathophysiology of CVDs and to design cell-specific epigenetic drugs that target cell subsets which only detrimentally contribute to disease progression. Another important challenge will be the correct timing of therapeutic intervention.

In addition to specific epigenetic inhibitors, further elucidation of the immune-metabolic basis of trained innate immunity, such as the metabolic shift that occurs, might provide alternative novel drug targets. Drugs that interfere with glycolysis (such as metformin, direct mTOR inhibitors, glycolysis inhibitors) prevent the occurrence of trained immunity in in vitro settings [67]. In animal models of neovascularization it has been revealed that glycolysis inhibition limits neovessel formation [107]. A number of small studies in patients with atherosclerosis have evidenced the systemic anti-inflammatory effect of metformin [108]. In addition, metformin inhibited monocyte to macrophage differentiation, and reduced pro-inflammatory cytokine production [109]. However, a real anti-atherosclerotic effect by metformin administration has not been proven yet. Although metformin treatment reduces cardiovascular morbidity and mortality in patients with diabetes compared to alternative glucose-lowering agents with similar glycemic control [110], metformin could not limit the intima media thickening in a recent trial in patients without diabetes [111].

## CONCLUSION

There is accumulating evidence that many cardiovascular risk factors, both traditional and non-traditional (e.g. nutrients, microbiota), induce a long-term (epigenetic) reprogramming of cells of the innate immune system, which in turn may provoke a condition of continuous innate immune cell activation. It still remains an open question, which cellular processes act to initiate or maintain these alterations. An increasing number of studies support the idea of a close connection between systemic and cellular metabolic processes and epigenetic reprogramming in the induction of trained innate immunity. These findings pave the road for the identification of novel drug targets. Further mechanistic insights on the induction of trained innate immunity are necessary to make beneficial use in future clinical therapeutic applications. Especially, unraveling the mechanisms in the various stages of atherosclerosis will be a challenging task within the next years. Nevertheless, epigenetic remodeling and the use of 'epigenetic drugs' are a promising tool for future treatment of atherosclerosis.

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General discussion

Nederlandse samenvatting

Dankwoord

List of publications

Curriculum Vitae



# CHAPTER 9

GENERAL CONCLUSION, DISCUSSION AND FUTURE  
PERSPECTIVES

Despite the discovery of several new and effective lipid-lowering drugs, cardiovascular disease (CVD) is still the leading cause of death worldwide<sup>1</sup>. The main underlying pathological process of CVD is atherosclerosis. This has long been considered a vascular lipid storage disease, but is now characterized as a chronic inflammatory disorder of the arterial vessel wall. Ultimately, atherosclerosis can lead to complete vascular occlusion, myocardial infarction or stroke<sup>2,3</sup>. There is a strong need to better understand the inflammatory mechanisms in atherosclerosis, to identify novel drug targets that can be used in the prevention and treatment of atherosclerosis. Therefore, in this thesis, we aimed to explore a possible hypothesis for the cause of persistent inflammation in atherosclerosis. A recent groundbreaking discovery in immunology is that cells of the innate immune system are also able to build a long-term immunological memory. This phenomenon has been named ‘trained immunity’. In this thesis we propose that trained immunity contributes to the development of atherosclerosis and provide several lines of evidence to confirm this hypothesis.

The general view that only the adaptive immune system can build an immunological memory has recently been challenged<sup>4</sup>. In organisms lacking adaptive immunity such as plants and invertebrates, the innate immune system can build up resistance against reinfection. Interestingly, recent studies have shown that also in vertebrates, innate immune cells display persistent adaptive characteristics leading to non-specific memory, which has been termed ‘trained immunity’<sup>5,6</sup>. It is likely that in evolution, trained immunity evolved as a primitive form of immune memory, aimed to provide additional protection of the host against reinfection. This could increase survival, especially in the newborn, who lacks a mature and functional adaptive immune system. However, there may also be a dark side to the reprogramming of innate immunity, in which increased inflammatory responses towards exogenous or endogenous stimuli could have deleterious effects, such as atherosclerosis<sup>7</sup>.

This thesis describes a series of *in vitro* and *in vivo* experiments in which the role of trained innate immunity in atherosclerosis is explored. First of all, we used a well-established *in vitro* model of trained immunity to investigate whether endogenous pro-atherogenic substances, such as oxLDL and lp(a) can induce trained immunity. Secondly, the role of trained immunity was explored in two *in vivo* studies of patients with or without atherosclerosis. We translated our findings of oxLDL training *in vitro* to an *in vivo* study of patients with elevated levels of lipoprotein(a), a carrier of oxidized phospholipids in the circulation. Next, we studied the inflammatory and epigenetic phenotype of patients with symptomatic and asymptomatic atherosclerosis and matched healthy controls. Thirdly, we studied the role of the cholesterol synthesis pathway in trained immunity *in vitro* and pharmacological inhibition of this pathway using statins, a well-known drug for the treatment of CVD.

## Part One: In vitro trained innate immunity and atherosclerosis

In chapter 3, we validated an in vitro model of trained immunity. We observed that 24 hours of training and 6 days of resting is the most optimal condition for inducing a trained immunity phenotype in monocyte-derived macrophages. We used this model in chapter 4, where we showed that trained immunity can be induced by modified LDL (oxLDL as well as acetylated LDL), but not by native LDL<sup>8</sup>. Twentyfour hour exposure of human monocytes to low concentrations of oxLDL resulted in an increased production of pro-atherogenic cytokines and chemokines (such as IL-6, TNF $\alpha$ , IL-8, and MCP-1) upon re-stimulation of the cells 7 days later with TLR4 or TLR2 agonists. Additionally, mRNA expression of these and other atherogenic cytokines and proteases was significantly higher in the trained monocytes 6 days after the initial stimulus. Furthermore, trained monocyte-derived macrophages displayed increased foam cell formation with higher expression of the scavenger receptors CD36 and SR-A and decreased expression of the cholesterol efflux transporters ABCA1 and ABCG1. Pathway analysis revealed that oxLDL-induced training is dependent on TLR2 and TLR4 activation, and activation of extracellular regulated kinase (ERK) and phosphoinositide 3 kinase (PI3K). Further laboratory analyses revealed that oxLDL-induced trained immunity was mediated via epigenetic reprogramming at the level of histone methylation. Brief stimulation of human monocytes with oxLDL led to long-term enrichment of H3K4 trimethylation, which is a prototypical long-term activating epigenetic mark, on the promoters of pro-inflammatory cytokines and chemokines. Moreover, oxLDL induced trained innate immunity was completely prevented by co-incubation with a nonspecific histone methyltransferase inhibitor.

For a translation of our observation to patients with atherosclerosis, it is important to consider the evidence that oxLDL is present in the circulation of patients with (an increased risk for) atherosclerosis. In other words, does oxLDL stimulate monocytes in the circulation in humans in vivo? It is generally believed that it is not possible to have high levels of oxLDL in the circulation for a long period of time. Main reasons are that the blood is rich in antioxidants, and it is rapidly cleared by the liver<sup>9</sup>. Furthermore, generally all vertebrates have preexisting circulating autoantibodies against oxLDL, that rapidly remove any oxLDL in the circulation. On the other hand, strong evidence does exist that smaller but detectable levels of modified LDL are present in the circulation<sup>10</sup>, for example in patients with metabolic syndrome, obesity, diabetes and CVD<sup>11</sup>. In obese children and adolescents, serum oxLDL levels are higher than in healthy controls, and interestingly, oxLDL levels decrease again upon strong weight loss<sup>12</sup>. In patients with diabetes, circulating levels of oxLDL were significantly higher than in control patients without diabetes<sup>13</sup>. In patients with CVD, the concentration of circulating minimally modified LDL (mmLDL) is associated with the severity of CVD. For example, plasma levels of



mmLDL are considered as a prognostic indicator of mortality in patients with heart failure<sup>14</sup>. Ehara et al. showed that plasma levels of mmLDL were significantly elevated in patients with CAD compared to a matched control group<sup>15</sup>. Furthermore, Holvoet et al. showed that LDL containing oxidation-specific epitopes could differentiate normal patients from patients with CAD, transplant atherosclerosis patients and acute coronary syndrome (ACS)<sup>16,17,18</sup>. More importantly, Tsimikas et al. showed that plasma oxLDL levels temporarily rise after an ACS with 35% in the first month after the event<sup>19</sup>. This is of special interest for our hypothesis, since a myocardial infarction (MI) is known to increase the risk for future events, by increasing the inflammatory response of monocytes and monocyte levels in the circulation<sup>20</sup>.

### Part Two: In vivo trained innate immunity and atherosclerosis

Although the role for circulating oxLDL in the pathogenesis of atherosclerosis is still a matter of debate, this is better established for other circulating lipoproteins which carry oxidized phospholipids, such as lipoprotein(a) [Lp(a)]. In chapter 5, together with our collaborators from the AMC, we conducted a series of in vitro and ex vivo studies to investigate whether Lp(a) can induce trained innate immunity. We showed that human isolated Lp(a) can induce a trained immunity phenotype in vitro in healthy monocytes, using the in vitro trained innate immunity method from chapter 3. Ex vivo analysis of circulating monocytes from patients with extremely elevated Lp(a) levels showed a comparable pro-inflammatory phenotype, which is characterized by increased pro-inflammatory cytokine production and increased expression of activation markers on the membrane compared to monocytes isolated from control patients with normal Lp(a) concentrations. Furthermore, the expression of adhesion molecules, such as CCR7, was higher, which was accompanied with an increased binding and migration on cultured endothelial cells in vitro. Lp(a) is comprised of an LDL particle, which is covalently bound to an apo(a) particle, which subsequently carries oxidized phospholipids. Further mechanistic studies showed that not the apoB, but the apo(a) particle of Lp(a) induced the training effect, and in more detail the oxidized phospholipids. When the oxPLs on apo(a) were blocked using a blocking antibody, the trained immunity phenotype was diminished. Our collaborators from Amsterdam have performed in vivo imaging studies on the subjects with elevated Lp(a) levels, that clearly demonstrate the potential implications of the pro-inflammatory monocyte phenotype for atherosclerosis. First, monocytes from patients and controls were labeled with 99-Tm and reinfused in the subjects. The cells were tracked in vivo by use of SPECT-CT imaging. We showed an increased accumulation of monocytes from patients with elevated Lp(a) in the arterial wall, whereas monocytes from healthy controls did not accumulate in the arterial wall. Finally, we also showed increased arterial wall inflammation in patients with elevated Lp(a), measured by FDG-PET/CT, both in the aorta and

carotid arteries.

What we didn't show in chapter 5 is whether this trained innate immunity phenotype is dependent on the same epigenetic and metabolic changes as previously shown for oxLDL and  $\beta$ -glucan. This will be topic of future studies. An important clinical question that remains is whether pharmacological strategies that lower Lp(a), such as antisense oligonucleotides targeting apolipoprotein(a) or PCSK9 inhibitors, could reverse the inflammatory phenotype of the monocytes.

The lifespan of monocytes is limited to only hours to days, while the trained immunity phenotype persists much longer in the circulation. Therefore, future studies should also comprise whether elevated levels of Lp(a) can induce changes in the monocyte progenitor cells. As shown by van der Valk et al., oxLDL can induce increased myeloid skewing and increased CCR2 expression on HSC-derived monocytes<sup>21</sup>. Whether or not Lp(a) can also induce these effects, is currently not known.

In conclusion, in chapter 3,4 and 5 we have shown that several cardiovascular risk factors and pro-inflammatory stimuli can induce trained innate immunity. The next approach was to study whether monocytes from patients with established atherosclerosis show a trained immunity phenotype, both in terms of cytokine production, as well as in terms of epigenetic and metabolic reprogramming. In chapter 6, we selected two groups of patients with atherosclerosis, one with symptomatic coronary atherosclerosis, and the other with asymptomatic carotid atherosclerosis, together with two matched control groups. We isolated circulating monocytes and extensively phenotyped these cells in terms of ex vivo cytokine production. We studied both epigenetic changes as well as metabolic reprogramming as underlying mechanism for increased inflammation. We demonstrated that circulating monocytes of patients with symptomatic atherosclerosis have a pro-inflammatory phenotype. This was associated with epigenetic remodeling at the level of histone methylation and increased expression of rate limiting enzymes of the glycolysis pathway and the pentose phosphate pathway. Interestingly, this pro-inflammatory phenotype was present only in patients with severe symptomatic coronary atherosclerosis. Patients with mild asymptomatic carotid atherosclerosis did not show a pro-inflammatory phenotype.

We studied the RNA expression profile of immune cells in PAXgene blood, which contains all inflammatory cells. In these samples, there was an increase in pro-inflammatory gene expression. Interestingly, also the gene expression of important rate-limiting glycolytic enzymes was increased, which was correlated to the increased pro-inflammatory response. This suggests that not only in plaque macrophages, intracellular metabolism is switched to increased glycolysis and correlates to increased inflammation as has been shown before<sup>22</sup>,

but that this could also be detected in circulating cells. Unfortunately, we only stored PAXgene tubes for RNA analysis and did not store CD14+ selected monocytes. Therefore, we can only speculate on the origin of cells that have both increased glycolysis and increased inflammation. In future studies, this will certainly be a topic of interest.

To our surprise, the analysis of epigenetic marks showed lower H3K4me3 on pro-inflammatory genes in patients with established atherosclerosis compared to controls. Lower activating marks but higher gene expression could only mean that there should also be lower repressive marks present, such as H3K27me3 and H3K9me3. Indeed, analysis of the repressive marks H3K27me3 and H3K9me3 showed profound enrichment of these marks on the promoter region of TNF $\alpha$ . H3K27me3 on TNF $\alpha$  correlated negatively with TNF $\alpha$  RNA expression, whereas there was no correlation with H3K4me3, indicating that repression rather than activation marks in these patients drive the pro-inflammatory response. It would be of great interest to perform whole genome ChIP-sequencing on these samples to see which pathways are up- or down-regulated in levels of activating or repressive histone marks. Also, it would be of interest to study more histone marks. Unfortunately, lack of sample prevented us from doing this.

One important fact complicating this story is the difference in medication use between the symptomatic patients and controls. Almost all patients were taking statins and controls were mostly statin-free. Interestingly, statins are known to decrease the inflammatory response (as explained in part three) and these patients still had an elevated inflammatory response. Therefore, this will not explain the differences observed regarding cytokine production and statins might even dampen the original effect. However, statins are known to induce changes in epigenetic marks, such as H3K27Ac but also H3K4me3, H3K27me3 and H3K9me3<sup>23–27</sup>. We therefore cannot exclude the influence of statins on the results in this study. In future studies, we will need to study the effect of statins on epigenetic marks in patients in vivo.

Although levels of histone methylation and acetylation have been studied in plaques before<sup>28–30</sup>, only two studies have studied the association between histone marks in circulating cells and CVD. First, Gómez-Uriz et al. showed that in patients with or without stroke, H3K4me3 and H3K9Ac did not significantly differ in the total blood cell population, although there seemed to be a trend for increased H3K4me3 in stroke patients compared to control<sup>31</sup>. More striking was the correlation between H3K4me3 and H3K9Ac levels and the levels of circulating TNF $\alpha$ . In our study, we found a negative correlation between H3K27me3 and TNF $\alpha$  RNA expression levels, as well as a correlation between H3K4me3 and H3K27me3. This indicates that epigenetic remodeling in patients with CVD is associated with levels of pro-inflammatory markers. Secondly, Shen et al. showed that in patients with acute ischemic stroke, levels

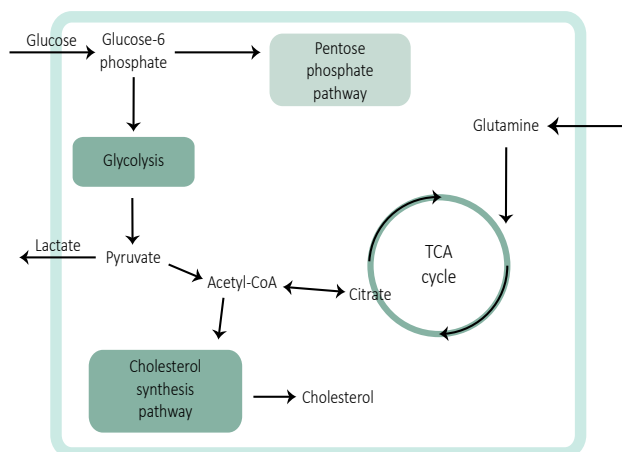
of acetylated histone H3 in PBMCs were lower than in healthy controls. Unfortunately the authors did not explain any possible mechanism<sup>32</sup>. Where DNA methylation in circulating cells in CVD are widely studied<sup>33</sup>, there is a great gap in studies to histone modifications in circulating cells in relation to CVD. It is believed to be an important epigenetic mechanism involved. In the future, we hope to add new insights in this mechanism. We have seen that trained immunity in vitro does not completely translate to the in vivo situation in patients with atherosclerosis. Different risk factors might induce different epigenetic changes. A broader study to histone modifications in all risk factors for atherosclerosis will be necessary.

When the results of both in vivo studies are combined, we might hypothesize that trained immunity does not play a role in the initiation of atherosclerotic plaques, but rather in the progression and destabilization. This fits with the observation in previous clinical studies that elevated lipoprotein(a) is not associated with early atherosclerotic lesions (e.g. carotid IMT) whereas elevated lp(a) is consistently associated with cardiovascular events, which mostly result from vulnerable atherosclerotic plaques<sup>34–36</sup>. We have also shown that in asymptomatic subjects with plaques on carotid ultrasound, there is no increased inflammation present in plasma or in monocytes. In patients with more severe symptomatic coronary atherosclerosis as well as in patients with elevated levels of lp(a), however, a pro-inflammatory phenotype can be clearly detected.

### Part Three: Pharmacological intervention and trained innate immunity

#### The cholesterol synthesis pathway in trained immunity

The induction of trained immunity is mediated by activation of both immune and metabolic pathways that results in epigenetic rewiring of cellular functional programs<sup>7,37,38</sup>. Using analysis of transcriptomics and metabolomics data, several pathways were identified as indispensable for the induction of trained immunity by  $\beta$ -glucan in monocytes (Figure 1)<sup>39</sup>. Interestingly, in  $\beta$ -glucan trained



**Figure 1:** upregulated pathways in trained immunity in vitro  
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monocytes, the cholesterol synthesis pathway was significantly upregulated. In chapter 7, we explored the role of the cholesterol synthesis pathway in trained immunity, using two different approaches. First, we inhibited the cholesterol synthesis pathway in the in vitro trained immunity model of chapter 3 with statins and other inhibitors of the pathway more downstream. In vitro, preincubation of monocytes with fluvastatin before beta-glucan or oxLDL training prevented the increased cytokine production upon restimulation on day 6. Also, the switch to increased glycolysis as well as the increase in foam cell formation was inhibited. Further study revealed that the accumulation of intermediates in this pathway, in particular mevalonate, was responsible for inducing the training effects. Transcriptome and epigenome analysis showed that incubation of healthy monocytes with mevalonate alone mimicked the trained immunity phenotype. This indicates that next to fumarate<sup>39</sup>, mevalonate is an essential factor in the metaboloepigenetics of trained immunity. Secondly, we studied monocytes of patients with a genetic deficiency of mevalonate kinase. These patients suffer from the Hyper IgD Syndrome (HIDS), one of the many hereditary periodic fever syndromes. HIDS patients carry a mutation in mevalonate kinase that is causative for the clinical presentation, which is characterized by symptoms as febrile attacks, skin lesions, arthritis and lymphadenopathy. The mutation in mevalonate kinase leads to accumulation of mevalonate and results in elevated excretion of mevalonic acid during attacks, a process which can be diminished by statins. Since patients with HIDS accumulate mevalonate intracellularly, we hypothesize that monocytes from these patients could resemble a (partly) trained immunity phenotype. RNA sequencing and ChIP sequencing analyses showed that indeed monocytes from patients with HIDS also show a trained immunity phenotype, characterized by increased cytokine expression as well as an upregulated mTOR and glycolysis pathway.

The upregulation of the cholesterol synthesis pathway only partly explains the molecular mechanisms of trained immunity. Therefore, in inflammatory diseases characterized by a trained immunity phenotype, treatment with statins might be a promising pharmacological strategy. Statins, HMG-CoA reductase inhibitors, are a widely used drug to treat patients at risk for CVD, especially for the treatment of elevated LDL-cholesterol. In addition, statins are increasingly prescribed to patients with other systemic inflammatory diseases leading to increased arterial wall inflammation, such as elevated levels of remnant cholesterol or lipoprotein(a), or for example patients with ankylosing spondylitis. When patients with ankylosing spondylitis were treated with statins, the arterial wall inflammation measured by FDG-PET/CT significantly decreased upon treatment. Additional treatment to lower the other pathways increased in trained immunity would be needed as well as reversing the epigenetic changes induced through these pathways.

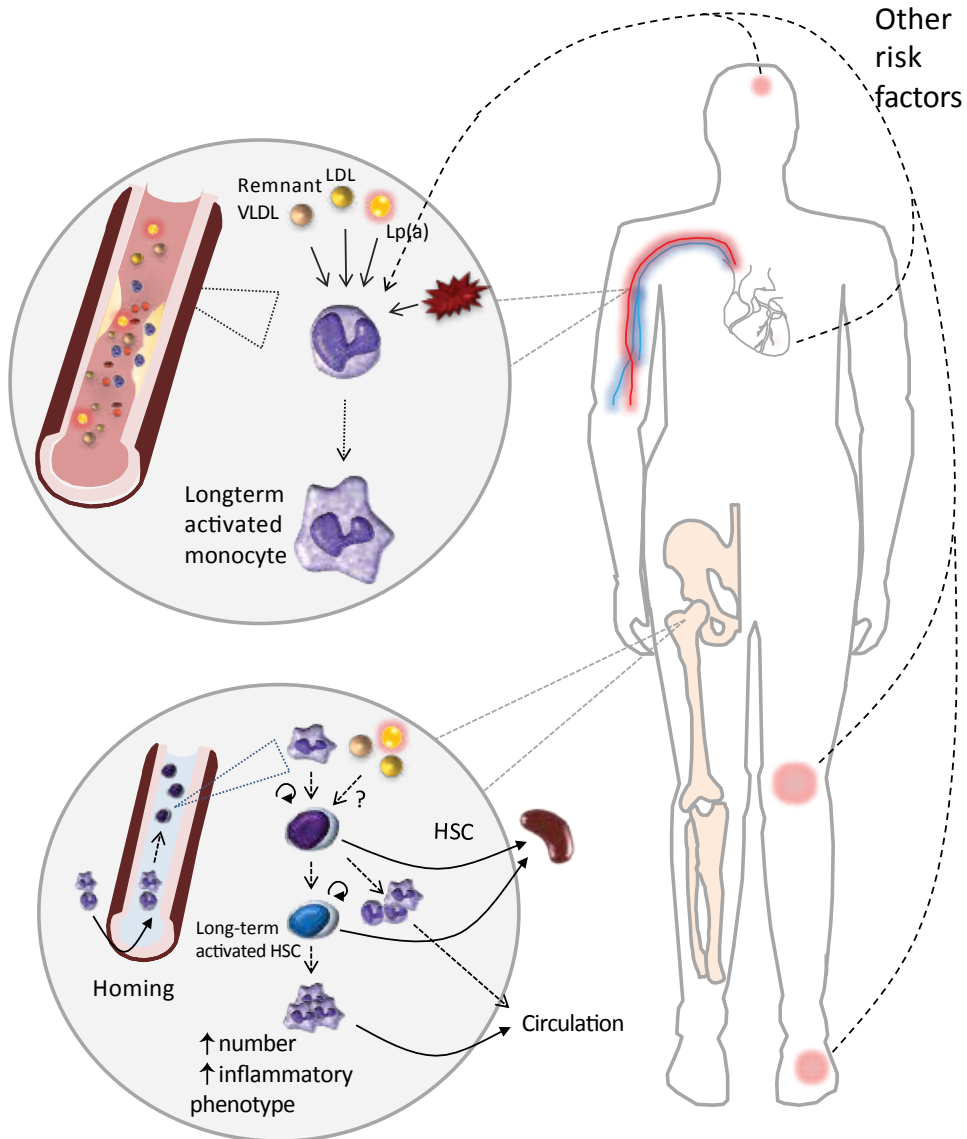
## FUTURE PERSPECTIVES

We now know that *in vitro*, in addition to the previously described PAMPs  $\beta$ -glucan and BCG, atherogenic DAMPs such as oxLDL and Lp(a) can induce trained innate immunity. *In vivo*, we have shown that monocytes from patients with elevated Lp(a) show a trained immunity phenotype and that monocytes from patients with symptomatic atherosclerosis show trained immunity by an increased pro-inflammatory phenotype and epigenetic and metabolic rewiring. However, the causal role for trained immunity in atherosclerosis still needs to be proven. Therefore, animal studies are needed that allow pharmacological modulation of the trained phenotype. For example, diet induced trained immunity as described in chapter 8 or infection. Also, the onset and progression of atherosclerosis should be studied. When trained immunity is indeed inducing increased atherosclerosis, the search for proper intervention will start.

Furthermore, the role of trained immunity in atherosclerosis in humans *in vivo* needs to be explored in the context of other traditional and non-traditional CV risk factors. Traditional risk factors should include diet and lipid-driven disorders, such as low levels of HDL, high triglycerides and elevated levels of LDL or other traditional risk factors such as previous events<sup>40,20</sup>. Examples of non-traditional risk factors comprise of chronic inflammatory diseases such as rheumatoid arthritis (RA), gout, ankylosing spondylitis, diabetes, HIV and other chronic or acute infections, which might share the common mechanism trained immunity<sup>41</sup>. A third layer in future studies to trained immunity in atherosclerosis will be the role of the bone marrow. As mentioned before, a trained immune phenotype can still be observed in circulating monocytes up to three months after vaccination of healthy subjects with BCG<sup>6</sup>. This period greatly exceeds the half-life of circulating monocytes, strongly suggesting that training already occurs at the level of the bone marrow progenitors. Indeed, in preclinical studies have already shown that the epigenome of myeloid progenitor cells can be modified by diet for prolonged periods of time, leading to increased atherosclerosis in mice<sup>42,43</sup>. Whether this also takes place in humans *in vivo* is topic of future studies (Figure 2). Subsequently, the mechanism of trained immunity needs to be elucidated in more detail to be able to construct biomarkers that might facilitate personalized medicine and to develop novel drugs for treatment or prevention of established atherosclerosis.

### Histone modifications as biomarkers in personalized medicine

How can the increasing knowledge about the role of epigenetic reprogramming in the pathophysiology of atherosclerosis be exploited to improve patient care? Recent developments in the fields of oncology and hematology offer a glimpse of the various possibilities. For example, the presence of specific histone modifications can be used to predict the outcome



**Figure 2:** the induction of trained immunity by traditional and non-traditional risk factors for CVD and atherosclerosis ©Siroon Bekkering

in selected patient groups. This was first described for patients with prostate carcinoma<sup>44,45</sup>, but recently also in much more cancer types<sup>46</sup>. Here we can learn from the oncology field, since the amount of studies to the role of histone modifications in disease and as biomarker is greatly higher than the amount of studies in the cardiovascular field. In future, one can dream about being able to study patterns in the epigenetic marks and predict outcome based on histone modifications and design a personalized treatment strategy, making use of a wide

range of epigenetic drugs available.

#### Pharmacological intervention

Most importantly when studying epigenetic remodeling is that the enzymes that act as the ‘writers’ (DNA and histone methylases and acetylases) and ‘erasers’ (DNA and histone demethylases and deacetylases) of the epigenetic code are amenable to pharmacological modulation. This offers a potentially important novel target for pharmacotherapy<sup>47,48</sup>.

A few nonselective inhibitors of HDAC’s and of DNA methyltransferases are already in use in regular patient care in oncology and haematology<sup>47,48</sup>. In preclinical studies, nonselective HDAC inhibitors have been shown to reduce inflammation and disease severity in animal models, for example for arthritis, septic shock and inflammatory bowel disease<sup>64</sup>. Preclinical proof of the concept that ‘epigenetic drugs’ can also affect the development of atherosclerosis was provided by Choi et al in LDLr<sup>-/-</sup> mice<sup>65</sup>. Treatment with the nonselective HDAC inhibitor trichostatin A increased atherosclerotic lesion size, and increased macrophage accumulation in aortic sinus<sup>65</sup>. This finding contradicts the beneficial effects of myeloid HDAC3 deletion on atherosclerosis formation<sup>58</sup> and highlights two important issues in epigenetic drug development. Firstly, most writers and erasers have many difference isoforms that can have opposite effects. Secondly, similar isoforms can have opposing effects in different cell types: genetic deletion of HDAC3 in myeloid cells is associated with larger, but more stable atherosclerotic lesions<sup>20</sup>, whereas deletion of this similar isoform in endothelial cells enhances atherosclerosis development<sup>55</sup>. Therefore, techniques that enable cell-specific or plaque specific targeting of drugs, such as nanoparticles, will facilitate optimal future use of epigenetic drugs<sup>66-68</sup>. For example, Duivenvoorden et al used reconstituted HDL particles to directly target the atherosclerotic plaque macrophage and increase the potency of anti-inflammatory statin therapy<sup>69</sup>. They showed that statin-loaded recombinant HDL particles accumulate in atherosclerotic lesions in which they specifically target plaque macrophages, and thereby inhibit inflammation and plaque progression. Now we know how important the role of the cholesterol synthesis pathway is in trained immunity, locally delivering statins to plaques becomes even more attractive. Very recently, Zheng et al have performed preliminary studies to the therapeutic options of treatment in humans in vivo with this nanoparticle. In humans in vivo, labeled rHDL targets the plaques in the arterial wall, whereas plaque-free areas were free of rHDL<sup>49</sup>. These clinical findings may guide future nanomedicine development using HDL particles for drug delivery in atherosclerosis in humans.

Next to the difficulties in developing specific histone acetylation inhibitors, targeting histone methylation is an even greater challenge. To date, 28 lysine methyltransferases have been identified that catalyze histone methylation<sup>62</sup>. These enzymes generally target specific lysine



residues and can either promote or repress gene transcription, depending on the specific lysine residue involved. In contrast to HDAC inhibitors and DNA methyltransferase inhibitors, (selective) histone methyltransferase inhibitors are not yet available for use in humans. Therefore, one of the challenges of the next decade is the development of more specific inhibitors of epigenetic writers and erasers, as well as reliable techniques that allow for targeting of specific cell types, such as monocytes and macrophages. Only with the availability of these methodologies it will be possible to use the exciting properties of trained immunity to optimize pharmacotherapeutic strategies for patients with (risk factors for) cardiovascular diseases.

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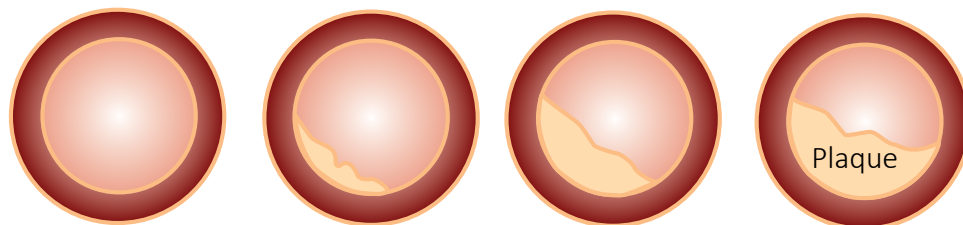




# NEDERLANDSE SAMENVATTING

## Atherosclerose, ofwel aderverkalking

Atherosclerose, vroeger aderverkalking genoemd, is een chronische ontstekingsziekte van de vaatwand. Atherosclerose ontstaat doordat witte bloedcellen cholesterol opeten. De witte bloedcellen kunnen dit niet goed verwerken, waardoor ze gaan plakken aan de binnenkant van de bloedvaten. De witte bloedcellen zorgen dan voor een ontsteking. Ze produceren stoffen die nog meer witte bloedcellen aantrekken, waardoor ze zich gaan stapelen. Er ontstaat een plaque (figuur 1).



*Figuur 1: overzicht van de verschillende fases van atherosclerose @Siroon Bekkering*

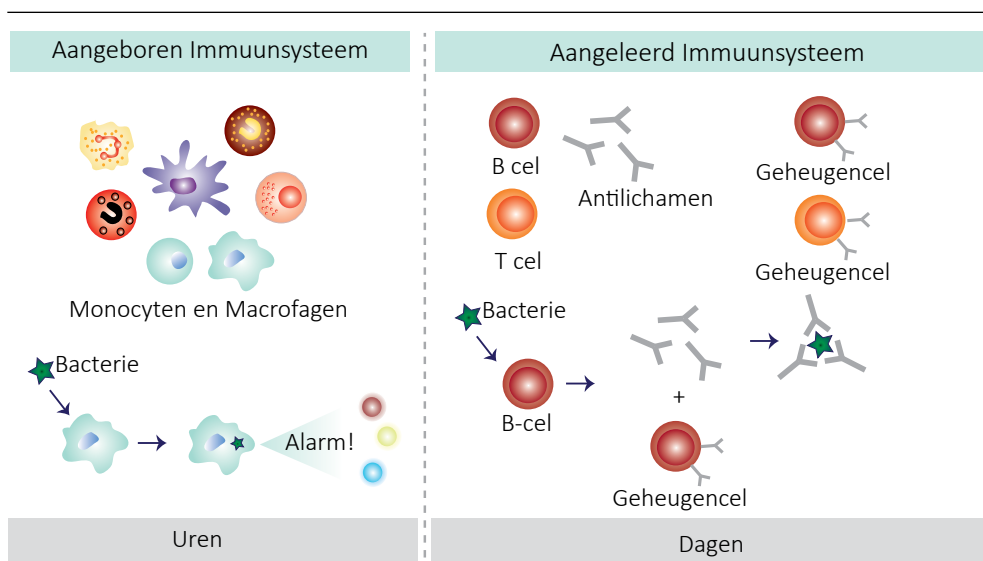
We weten dat witte bloedcellen die rondzwemmen in het bloed bij patiënten met atherosclerose veel actiever zijn dan witte bloedcellen van gezonde mensen. Deze witte bloedcellen gaan makkelijker door de wand van de bloedvaten heen. Ook maken ze sneller en meer ontstekingsstoffen. In mijn thesis heb ik onderzocht hoe de witte bloedcellen nu precies actiever worden en of we deze activiteit kunnen remmen.

## Twee immuunsystemen

Er zijn heel veel verschillende soorten witte bloedcellen (figuur 2). De witte bloedcellen die cholesteroleten heten monocyten of macrofagen horen bij het aangeboren immuunsysteem. Zoals de naam al zegt, heb je deze cellen al vanaf je geboorte, ze zijn aangeboren. Deze cellen werken niet heel specifiek, maar ruimen alles op wat lichaamsvreemd is. Ook hebben we een aangeleerd immuunsysteem met een geheugen. Dat geheugen bouw je elke keer op als je ziek bent of bijvoorbeeld met een vaccinatie. Dan krijg je een klein beetje ziekteverwekker, waarvan je niet ziek wordt, maar die je aangeleerde immuunsysteem wel onthoudt. Je lichaam maakt een geheugencel aan. De volgende keer dat je deze ziekteverwekker krijgt, ruimt je lichaam deze sneller op en word je minder ziek.

## Monocyten met geheugen

Tot vier jaar geleden dacht men dat cellen van het aangeboren immuunsysteem geen geheugen konden hebben. Nu blijkt dat anders te zijn: als monocyten een klein beetje lichaamsvreemde stof tegenkomen, gaan ze zich hierna anders gedragen, hyperactief. Dit is al aangetoond voor een aantal vaccins, die hierdoor beschermen tegen meer dan alleen



**Figuur 2:** Twee immuunsystemen werken verschillend in specificiteit en snelheid: het aangeboren immuunsysteem werkt snel (binnen een paar uur) maar aspecifiek: het ruimt alles op wat lichaamsvreemd is. Het aangeleerde immuunsysteem werkt via antilichamen, die worden gemaakt door B en T-cellen. Nadat deze cellen voor de eerste keer een antilichaam hebben gemaakt, wordt er een geheugencel gemaakt, die sneller werkt bij de volgende keer dat je ziek wordt ©Siroon Bekkering.

de ziekteverwekker waarvoor het vaccin gemaakt is. Dit geheugen is niet specifiek, zoals bij het aangeleerde immuunsysteem, maar de cellen gaan zich actiever gedragen tegenover alle nieuwe indringers.

### Mijn hypothese en onderzoek in het lab

In mijn thesis vroeg ik me nu af: 'Zien monocyten sommige vormen van cholesterol bijvoorbeeld als lichaamsvreemd en ontwikkelen ze hierdoor een vergelijkbare hyperactiviteit?' In de afgelopen jaren onderzocht ik deze hypothese op verschillende niveaus. Het eerste level is in het lab, in vitro noemen we dat. Ik gebruikte hiervoor monocyten uit het bloed van gezonde mensen, bijvoorbeeld van de bloedbank. Ik bracht monocyten van gezonde mensen in contact met verschillende vormen van cholesterol. Daarna keek ik of deze cellen zich dan anders gingen gedragen dan monocyten die in hun 'gewone' groeimedium werden gehouden, zonder cholesterol. Ik keek of ze meer ontstekingsstoffen gingen maken, en of de cellen dus hadden onthouden dat ze een paar dagen eerder met cholesterol in contact waren gekomen. En inderdaad, 6 dagen na het eerste contact met cholesterol waren de cellen nog steeds hyperactief. Ze maakten meer ontstekingsstoffen als ze daarna een bacterie tegenkwamen en gingen ook meer cholesterol opeten. Het leek wel alsof ze een geheugen hadden: na hun eerste ontmoeting met cholesterol leken ze in het volgende contact veel agressiever te reageren.



## Vlaggetjes op het DNA

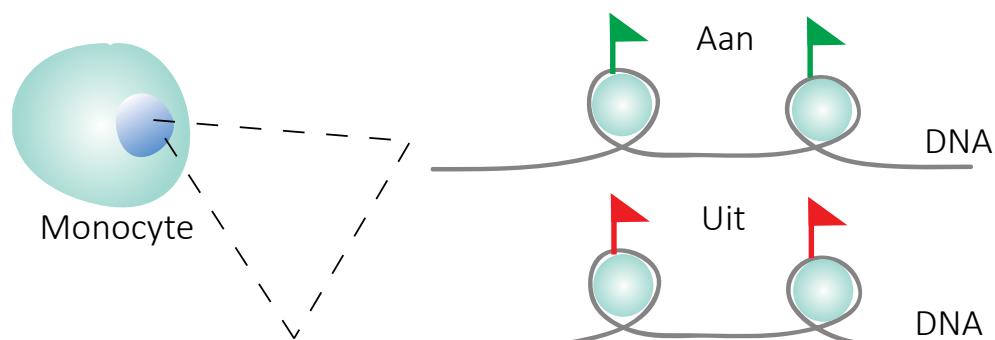
Je kan het geheugen zien als een soort vlaggetjes op het DNA. Er staan groene vlaggetjes op het DNA bij processen die aan staan in de bloedcel en rode vlaggetjes bij processen die uit staan (figuur 3). Zo weet de dirigent van de cel wat er wel en niet gemaakt moet worden. Maar als er te veel vlaggetjes staan, of als ze op de verkeerde plek staan, kan de cel hyperactief worden. Dan maakt het bijvoorbeeld te veel ontstekingsstoffen. De aanraking met cholesterol zorgt in deze monocytten voor het plaatsen van veel meer groene vlaggetjes. Dit betekent dat er veel meer eiwitten voor ontsteking in de cel gemaakt worden. Gelukkig zitten de vlaggetjes niet vastgelijmd, maar kunnen ze er ook weer afgehaald worden. Onderzoek hiernaar is dus veelbelovend, want er kunnen ook medicijnen worden gemaakt.

## Onderzoek in patiënten

In het tweede deel van mijn thesis ging ik ook kijken naar de monocytten van patiënten met hart- en vaatziekten. Dit deed ik in twee verschillende patiëntengroepen: in de eerste studie onderzocht ik patiënten die al veel plaques in hun aderen hebben en gezonde controles die dat niet hebben. Uit het bloed van deze mensen haalde ik de witte bloedcellen en ik onderzocht de (hyper)activiteit: hoeveel ontstekingsstofjes maken de witte bloedcellen van mensen met en zonder plaques? Daarna onderzocht ik het vlaggetjespatroon van de witte bloedcellen. Ik ontdekte dat er verschillen waren tussen de patiënten en de gezonde controles. Een eerste hint dus dat dit geheugen en de vlaggetjes misschien wel een rol spelen in het verloop van de ziekte.

## Een speciaal soort cholesterol: lipoprotein(a)

De tweede patiëntenpopulatie die ik bestudeerde waren patiënten met verhoogd lipoprotein(a). Dit is een speciaal soort cholesterol, waarvan we weten dat het kan zorgen voor activatie van de monocytten. Maar hoe dit precies werkt, en of dit misschien ook werkt



**Figuur 3:** Vlaggetjes op het DNA in een cel bepalen of het gen aan staat (groene vlaggetjes) of uit (rode vlaggetjes). Zo kan de dirigent van de cel weten welke eiwitten er gemaakt moeten worden en welke niet ©Siroon Bekkering.

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via de vlaggetjes, dat wisten we nog niet. Ik bekeek hoe monocytten van gezonde mensen reageerden als ze met dit lipoproteïne(a) in aanraking kwamen. Net als in het eerste gedeelte van mijn thesis gingen de monocytten zich hyperactief gedragen. En in de patiënten was dit net zo! De monocytten kropen eerder door de vaatwand en zorgden voor meer ontsteking. We zijn helaas nog niet toegekomen aan het onderzoeken van het vlaggetjespatroon in deze patiënten, dus ik hoop dat dat snel in de toekomst gaat gebeuren!

In het derde deel van mijn thesis dook ik meer in de mechanismen van dit hele proces: hoe werkt dit? Wat is nou anders in deze cellen met hyperactiviteit? Welke processen spelen hierbij een rol? Daarop zal ik verder nu niet in gaan.

### **Het toekomstplaatje**

Uiteindelijk is het natuurlijk de droom om een persoonlijk vlaggetjespatroon te kunnen ontdekken in verschillende patiënten die risico hebben op hart- en vaatziekten. Hopelijk kan de patiënt dan persoonlijke medicijnen krijgen om de cellen weer in normale staat te krijgen, om het teveel aan vlaggetjes te verwijderen. Want dat is het mooie: je wil niet de functie van de witte bloedcellen helemaal platleggen, omdat zij ons eigenlijk moeten beschermen tegen allerlei lichaamsvreemde stoffen zoals bacteriën. Als je het teveel aan vlaggetjes eraf haalt, haal je alleen het hyperactieve deel weg, en blijft de cel alsnog zijn werk doen. Je drukt als het ware op de resetknop!

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## LIST OF PUBLICATIONS

1. Hoogeveen R, Nahrendorf M, Stroes ESG, Riksen NP, Netea MG, Joosten LAB, Neidhart M, Nordestgaard B, Catapano AL, **Bekkering S**. Reprogramming monocytes in atherosclerosis. Submitted (2017)
2. Verweij SL, van Duivenvoorden R, Nurmohammed NS, van der Valk FM, Versloot M, Verberne HJ, Stroes ESG, Nahrendorf M, Bernelot Moens SJ, **Bekkering S**. CCR2 expression on circulating monocytes is associated with arterial wall inflammation assessed by <sup>18</sup>F-FDG PET/CT. Submitted (2017)
3. **Bekkering S**, Arts RJW, Novakovic B, Kourtzelis I, Popa C, ter Horst R, van Tuijl J, Simon A, Stunnenberg H, Joosten LAB, Chavakis T, van der Meer JWM, Riksen NP, Netea MG. The mevalonate pathway drives metabolic and epigenetic reprogramming during induction of trained immunity. Submitted (2017)
4. Bernelot Moens SJ, Verweij SL, Schnitzler JG, Stiekema LCA, Bos M, Langsted A, Kuijk C, **Bekkering S**, Voermans C, Verberne HJ, Nordestgaard B, Stroes ESG, Kroon J. Remnant cholesterol elicits arterial wall inflammation and a multi-level cellular immune response in humans. *Arterioscler Thromb Vasc Biol* (2017) In press
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8. van der Valk FM, **Bekkering S**, Kroon J, Yeang C, Van den Bossche J, van Buul JD, Ravandi A, Nederveen AJ, Verberne HJ, Scipione C, Nieuwdorp M, Joosten LA, Netea MG, Koschinsky ML, Witztum JL, Tsimikas S, Riksen NP, Stroes ES. Oxidized phospholipids on Lipoprotein(a) elicit arterial wall inflammation and an inflammatory monocyte response in Humans. *Circulation*. (2016) 134(8):611-24
9. Van Dam AD, **Bekkering S**, Crasborn M, van Beek L, van den Berg SM, Vrieling F, Joosten SA, van Harmelen V, de Winther MP, Lütjohann D, Lutgens E, Boon MR, Riksen NP, Rensen PC, Berbée JF. BCG lowers plasma cholesterol levels and delays atherosclerotic lesion progression in mice. *Atherosclerosis* (2016) 251:6-14
10. Christ A, **Bekkering S**, Latz E, Riksen NP. Long-term activation of the innate immune system in atherosclerosis. *Semin Immunol* (2016) Apr 21.
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14. **Bekkering S**, Quintin J, Joosten LAB, van der Meer JWM, Netea MG, Riksen NP. Oxidized low-density lipoprotein induces long-term proinflammatory cytokine production and foam cell formation via epigenetic reprogramming of monocytes. *Arterioscler Thromb Vasc Biol* (2014) 34(8):1731-1738.
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## CURRICULUM VITAE

Siroon Bekkering werd op 26 augustus 1988 geboren in Houten. Na het behalen van haar Gymnasium-diploma aan het Stedelijk Gymnasium te Utrecht in 2005, deed ze een oriëntatiejaar aan de Evangelische Hogeschool in Amersfoort. Dat was maar goed ook: vóór dit oriëntatiejaar was zij van plan Griekse en Latijnse taal en cultuur te gaan studeren. Na het oriëntatiejaar wilde zij liever de bèta kant op.

In 2006 begon zij aan de studie Scheikunde aan de Radboud Universiteit in Nijmegen, maar na een half jaar switchte ze naar Moleculaire Levenswetenschappen (MLW). Tijdens haar bachelor deed ze een stage op de afdeling Experimentele Urologie onder supervisie van Gerald Verhaegh en behaalde haar bachelor in 2009. Tussen haar bachelor- en master-studie deed zij een bestuursjaar bij de Nijmeegse Studentenvereniging de Navigators (NSN).

In 2010 ging zij verder met haar master Molecular Life Sciences, specialisatie research. Haar eerste stage deed zij op de afdeling Laboratorium voor Kindergeneeskunde en Infectieziekten (LKI) op het Radboudumc onder supervisie van Dimitri Diavatopoulos, in samenwerking met de afdeling Medische MicroBiologie (MMB) op het UMC Utrecht onder supervisie van Susan Rooijackers. Hier deed zij onderzoek naar ontsnapping aan het immuunsysteem van de bacterie *Streptococcus Pneumoniae*. Haar tweede stage deed zij in Kopenhagen in het Rigshospitalet onder supervisie Niels Borregaard, naar de localisatie van het eiwit ADAM8 in de neutrofiel. In 2012 studeerde zij cum laude af.

Na haar studie begon zij aan haar PhD op de afdeling Interne Geneeskunde van het Radboudumc. Haar onderzoek is in deze thesis beschreven en gepresenteerd op vele nationale en internationale congressen. Haar onderzoek besloeg laboratorium werk, gecombineerd met patiëntenstudies. Siroon werkte samen met vele andere ziekenhuizen voor deze doeleinden, onder andere met het Academisch Medisch Centrum (AMC) te Amsterdam, het Erasmus MC te Rotterdam, het Leiden Universitair Medisch Centrum (LUMC) en het Canisius Wilhelmina Ziekenhuis (CWZ) te Nijmegen. Ze treinde het hele land door voor samples.

Na haar PhD ging zij begin 2016 aan de slag als post-doc bij de afdeling Vasculaire Geneeskunde in het AMC onder begeleiding van prof. Erik Stroes. Zij continueert hier haar onderzoek uit haar PhD naar de rol van geheugen in het aangeboren immuunsysteem bij mensen met hart- en vaatziekten. De samenwerking met het Radboudumc is nog steeds nauw.

Siroon woont samen met Thom in Utrecht.